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Food quality and food safety aspects during meat product processing

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iii. Contribution of authors

Assessment of microbial quality in poultry drinking water on farms in Austria

Mustedanagic A., Matt M., Weyermair K., Schrattenecker A., Kubitza I., Firth L. C., Loncaric I., Wagner M., and Stessl. B.

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Wagner, M.:	Conceptualization, writing – review and editing
Stessl, B.:	Conceptualization, funding acquisition, project administration, resources, supervision, data curation, writing – review and editing

Characterization of *Leuconostoc carnosum* and *Latilactobacillus sakei* during cooked pork ham processing

Mustedanagic A., Schrattenecker A., Dzieciol M., Tichy A., Thalguter S., Wagner and Stessl. B.

Mustedanagic, A.:	Conceptualization, methodology, formal analysis, investigation, data curation, visualization, writing and editing the manuscript
Schrattenecker, A.:	Investigation, data curation
Dzieciol, M.:	Methodology, review and editing the manuscript
Tichy, A.:	Statistical analysis
Thalguter, S.:	Data curation, review and editing the manuscript
Wagner, M.:	Resources, Review and editing the manuscript, supervision
Stessl, B.:	Conceptualization, methodology, formal analysis, resources, writing, review and editing the manuscript, supervision, project administration, funding acquisition

iv. Declaration

I confirm that I followed the rules of good scientific practice in all aspects.

v. Summary

English

The meat products are susceptible to contamination due to their inherent perishability. Therefore, the primary concerns regarding meat safety and quality concerns revolve around bacterial pathogens and process-related contaminants. This concern extends to assessing bacterial environmental quality and safety indicators arising from environmental and process-related aspects along the meat production chain.

This thesis utilized culture-based methods to assess the hygiene status of meat production environments at distinct stages: primary poultry production and cooked ham processing. The assessment included examining aerobic mesophilic counts, *Enterobacteriaceae, Pseudomonadaceae*, and *Campylobacter* spp. in poultry drinking water before and after sanitation of waterlines. The results revealed inadequate waterline management practices at poultry farms leading to high microbial loads and presence of opportunistic pathogens such as *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp. These bacteria pose potential health risks to humans, animals, and meat safety. *Campylobacter jejuni* was detected in one sample before waterline cleaning. It is possible that *Campylobacter* spp. may be present in poultry drinking waterlines in a viable but non-cultivable state, necessitating the use of special detection methods.

Investigations into the processing environment of cooked ham, in conjunction with limitations on the shelf-life of cooked ham, identified lactic acid bacteria as a primary constraint with no observable spoilage effects. Specifically, *Leuconostoc carnosum* and *Latilactobacillus sakei* were dominant species in cooked ham. Further strain-level investigation confirmed that prevalent strains during cooked ham storage were present in raw meat and the post-cooking area of the processing facility.

The insights gained during present thesis has been used to educate both poultry farmers and cooked ham production facilities, improving water management practices and cooked ham processing hygiene. The findings in this thesis trespass specific contexts

and are applicable across diverse meat production settings. They contribute to our understanding of food processing microbiota, aiding in minimizing contamination by spoilage organisms and potential pathogens across various food production environments.

German

Die Hauptbedenken hinsichtlich der Sicherheit und Qualität von Fleisch betreffen bakterielle Krankheitserreger sowie Verunreinigungen, die durch den Herstellungsprozess entstehen. Diese Bedenken resultieren aus der Anfälligkeit von Fleischprodukten für Kontaminationen und der damit verbundenen Verderblichkeit.

Diese Arbeit verwendete kulturelle Untersuchungsmethoden, um die Hygiene in der Geflügelproduktion und bei der Herstellung von gekochtem Schinken zu bewerten. Sie überprüfte die Anzahl von verschiedenen Mikroorganismen im Geflügeltrinkwasser vor und nach der Reinigung der Wasserleitungen. Die Ergebnisse zeigten unzureichende Reinigung der Wasserleitungen auf Geflügelfarmen, was zu hohen mikrobiellen Belastungen und dem Vorhandensein von opportunistischen Pathogenen wie *Pseudomonas* spp., *Stenotrophomonas* spp. und *Ochrobactrum* spp. führte. Diese Bakterien stellen potenzielle Gesundheitsrisiken für Menschen, Tiere und die Fleischsicherheit dar. Die eindeutige Identifizierung von Wasser als Hauptquelle für Kolonisierung von *Campylobacter* spp. in der Herde blieb aufgrund methodischer Einschränkungen unklar.

Bei Analysen der Produktionsumgebung, um die Faktoren zu ermitteln, die die Mindesthaltbarkeit von gekochtem Schinken beeinflussen, wurde festgestellt, dass Milchsäurebakterien eine Schlüsselrolle spielen, indem sie keine äußerlich erkennbaren Anzeichen von Verderb verursachen. *Leuconostoc carnosum* und *Latilactobacillus sakei* waren dominante Bakterienarten im Schinken, während Untersuchungen auf Stammenebene zeigten, dass die während der Lagerung vorherrschenden Stämme sowohl im rohen Fleisch als auch in der Verarbeitungsanlage nach dem Kochen vorhanden waren.

Die gewonnenen Erkenntnisse wurden genutzt, um die Wasserreinigung und Schinkenverarbeitung zu verbessern und wissenschaftliche Grundlage für behördliche Belastung zu verbessern. Sie tragen dazu bei, das Verständnis der Mikrobiota bei der Lebensmittelverarbeitung und zur Minimierung von Kontaminationen in verschiedenen Produktionsumgebungen bei.

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vi. Abbreviations

AMA	Agrar Markt Austria Marketing GmbH
AMC	Aerobic mesophilic count
CFU	Colony Forming Units
DS	Detergent-sanitizer
EB	Enterobacteriaceae
EFSA	European Food Safety Authority
EU	European Union
FPE	Food processing environment
GHP	Good Hygiene Practices
GRAS	Generally Recognized as Safe
HACCP	Hazard Analysis and Critical Control Points
ISO	International Standard Organization
LAB	Lactic acid bacteria
Lb. sakei	*Latilactobacillus sakei*
Leuc. carnosum	*Leuconostoc carnosum*
MAP	Modified atmosphere packaging
NGS	Next generation sequencing
PAA	Peroxyacetic acid
PCR	Polymerase chain reaction
PFGE	Pulsed-Field Gel Electrophoresis
PS	Pseudomonadaceae
QPS	Qualified Presumption of Safety
RTE	Ready-to-eat
VBNC	Viable but Non-Culturable
WGS	Whole genome sequencing
WHO	World Health Organization

1 INTRODUCTION

The consumption of meat has a crucial role in providing essential nutrients that sustain normal physiological functions, boost immunity, and prevent specific diseases, such as malnutrition. Meat is a rich source of proteins, vitamins, and minerals, which help alleviate nutritional deficiencies and promote human health [1].

The advancement of economy and improvement in living standards have contributed to increasing importance of meat and meat products [2]. Developing countries, accompanied by population growth and socio-economic progress, have witnessed a substantial rise in global meat consumption [3]. By the year 2050, the global human population will increase to 9.5 billion, leading to a twofold increase in the demand for meat and animal-based products [4]. This has driven the public and private food production sectors to establish higher standards for food safety and quality [5].

The safety and quality of the meat food supply is of primary importance worldwide. The most serious meat safety concerns are associated with microbial pathogens, especially bacterial pathogens [6]. The World Health Organization (WHO) estimated that over 600 million cases of foodborne illnesses were recorded in 2010, resulting in approximately 420.000 deaths [7].

The perishability and susceptibility to contamination of meat and meat products contribute to 23% of food loss and food waste within the meat sector [8]. This raises awareness among consumers and produces because of its health threat, economic and environmental impact [9,10]. The challenge of food losses is particularly prominent in the developing countries. This overproduction and waste of food coexist with the growing problem of hunger, creating an urgent global concern.

Achieving meat safety in a sustainable manner requires improved efficiency in the meat production system and reducing meat waste [11]. Compliance with standards and

regulations becomes imperative to address these concerns and ensure a positive contribution of livestock and meat production to human health while mitigating their negative impacts [12].

1.1 Current trends in meat production chain

Throughout the 20th century, shifts in meat production structures have altered veterinary public health and meat safety challenges. Specialization in livestock production, coupled with enhanced biosecurity measures like indoor animal rearing, application of Good Hygiene Practices (GHP) and hazard analysis and critical control points (HACCP) systems had significant importance for public health protection [13–15]. However, current trends in the meat production industry aim to merge preventive and control measures, emphasizing a comprehensive farm-to-fork approach [16]. This approach encompasses various factors influencing food safety risk management along the entire food chain. However, implementing these assessments is complex and resource-intensive, requiring extensive data and expert knowledge from diverse sources. Exposure assessments often concentrate on specific segments of the food chain, particularly those critical for implementing risk reduction measures or areas solely controlled by risk managers [16]. Notably, significant scientific and technological advancements are being made in poultry meat production to reduce biological hazards and/or their toxins at farms. Strategies to minimize pathogen prevalence in poultry include biosecurity measures, enhancing host resistance, implementation of efficient detection methods, and vaccination programs [14,17,18]. Nevertheless, contamination during post-processing within factories may serve as the primary source for introducing foodborne hazards, especially evident in ready-to-eat (RTE) pork foods associated with widespread environmental contamination [19,20]. In such cases, the farm-to-fork risk assessment concentrates specifically on events occurring after processing [16].

Moreover, current research studies emphasize the interplay between foodborne pathogens and the processing environment, particularly in poultry and pork meat production sector, which are two most consumed meat types worldwide [21]. Consequently, this thesis zooms in on safety risk assessment as the critical point in managing the safety risks associated with waterline sanitation in primary production withing the poultry section. This is considered a primary focus in ongoing research strategies. Additionally, the thesis investigates the assessment of contamination events within post-processing facility of cooked ham RTE product, recognizing these as crucial for managers to control.

1.2 Poultry and pork meat safety and quality concerns

The meat safety and quality is defined by intrinsic and extrinsic characteristics of meat, which ensure both safety and acceptability by the consumers [22]. Intrinsic traits include technological aspects like water holding capacity, texture, color, and sensory attributes, while extrinsic characteristics involve microbial loads, physical properties, and chemical attributes, all of which are essential for meat product safety and quality [23–25].

In food microbiology, the bacteria of interest can be classified into infectious agents, causative agents of foodborne intoxication, agents of spoilage, and processing-related bacteria [26]. Some of these bacteria hold technological significance in improving sensory (organoleptic and rheological) attributes of meat products [27]. Nevertheless, the presence of undesired bacteria, commonly referred as spoilage bacteria, can diminish meat quality and in more severe cases presence of pathogenic bacteria can compromise meat safety [28,29]. With more than 200 known diseases being transmitted through food, bacterial pathogens are gaining significant importance [30,31]. For example, in 2017, European Union (EU) member states reported a total of 5079 foodborne and waterborne outbreaks, with 60% attributed to bacterial pathogens present in meat and

meat products [32]. Similarly, in 2018, the EU reported 359.692 cases of foodborne and waterborne diseases, with a significant majority linked to meat consumption. Poultry meat consumption is frequently associated with reported foodborne outbreaks [33]. Consequently, ensuring microbiologically safe poultry meat requires coordinated efforts across all sectors, including poultry producers, processors, distributors, and retailers. Additionally, drinking water used in poultry production plays a crucial role in preventing and controlling bacterial infections within the One Health spectrum. It also contributes to improving antibiotic use and preventing the spread of antibiotic resistance [34]. This study specifically focuses on assessing the microbiological safety risks associated with waterline sanitation practices on poultry farms in Austria.

Simultaneously, consumers increasingly seek food products with improved sensory quality, enhanced functionality, nutrition, and a traditional, wholesome image, while also demanding safety, minimal processing, fewer additives, and greater convenience [35]. The high demand of RTE foods, derived from either fresh or pre-cooked sources, has risen due to limited time available for food preparation in households [36]. The safety and quality of RTE products is strictly linked to compliance with fundamental hygiene protocols, which are crucial for both immediate consumption and shelf-life. However, preventing contamination during processing of RTE products remains a growing challenge. Therefore, this thesis estimated the presence of process-related bacteria during the production and storage of RTE pork product in accordance with guidelines for acceptable microbial quality limits.

Microbial contamination during animal production and meat processing plants plays a crucial role in food quality and safety [37]. The physicochemical properties of meat provide favorable conditions for the colonization and growth of various microorganisms [38,39]. Contamination of meat can occur during slaughtering, through water, air, soil, workers, and equipment during meat processing stages [40–42]. During subsequent processing steps such as handling, cutting, and storage, abiotic factors including temperature, atmospheric conditions, pH, and NaCl levels promote the growth of

specific microbiota, leading to the colonization of meat by pathogens and/or spoilage-related bacteria species [39,43].

In an effort to reduce the incidence of foodborne diseases and to adapt to changing consumer preferences for freshness and convenience, while maintaining safety and extending shelf-life, a proactive approach has been introduced in industry-based settings to monitor foodborne pathogens and spoilage microorganisms throughout the entire meat production process [30]. This shift within the industry has been underpinned by extensive research aimed at enhancing hygiene standards and systematically incorporating food safety practices. Therefore, this study was conducted in collaboration with poultry producers and cooked ham production facility in Austria. The primary objective was assessment of specific hygiene practices in meat production while addressing meat safety and quality measures within the industry.

1.3 Poultry and pork meat production environment: A complex microbiological niche

Awareness of residential microbiota in food production environments is critical for multiple reasons [44]. Firstly, these bacteria can jeopardize food quality as they possess traits that enable their survival and growth, potentially leading to contamination during storage. Since production environments often share similar conditions with the food products, such as temperature, nutrients, and stress factors, processing surfaces can become reservoirs for spoilage bacteria [45]. Secondly, studies have indicated that nonpathogenic bacteria in the processing environment can influence the fate of introduced pathogens, affecting food safety outcomes [46]. Lastly, similar to other ecological niches, residential bacteria are suspected to contribute to the persistence and spread of antimicrobial resistance genes [34,47].

Contamination risks in the food production chain span from animal farming to final meat processing, involving risk factors such as unhygienic surfaces, poor handling, and inadequate meat processing practices [5].

At animal farms, pathogens can enter animal herds through various routes, including farming environment and equipment [48]. The pathogens naturally occur in the digestion tract of wild and farmed animals, soil, water, and plants. Farm animals can acquire microbial pathogens through grazing, contaminated feed, or water supplies, and contaminated farm equipment [49]. Additionally, shared animal feeders and water drinkers can also facilitate cross-contamination and contribute to animal infections. Once introduced to the farm environment, pathogens can survive for long periods on substrates such as feces, soil, water, building materials, and farming equipment. Moreover, animals can carry infections without exhibiting symptoms, functioning as asymptomatic carriers of human pathogens. This scenario poses a significant risk of contaminating holding areas and potentially transmitting infections to other animals through the fecal-oral route [50]. These asymptomatic carriers also elevate the likelihood of introducing pathogens into post-processing environments [51]. Additionally, the administration of antibiotics for disease treatment and prevention in animal production has been linked to the proliferation of antibiotic-resistant microorganisms [48].

When meat arrives at the processing plant, the meat production chain faces a new set of challenges related to meat quality and safety due to process-related contamination. In meat processing facilities, organic residues from meat on surfaces create an optimal environment for microbial growth and aggregation, significantly contributing to cross-contamination during meat processing conditions [52–54]. Bacterial pathogens and spoilage organisms exhibit better survival rates under dirty, humid, and cold conditions compared to clean, dry, and warm conditions [55]. As meat enters the processing facilities, these pathogens exhibit broad ranges of growth and survival mechanisms, allowing them to withstand the various hurdles within the food processing environment

(FPE) and proliferate in food products [37]. Their ability to adapt and survive in different environments represent a significant challenge for controlling the in both primary production and processing environment. Therefore, understanding and characterizing the microbiota during animal production and meat processing environment is critical in improving meat product quality and safety.

1.4 Foodborne pathogens and the safety of poultry meat production chain

The most recognized foodborne pathogens associated with meat consumption include *Campylobacter* (*C.) jejuni* and *C. coli*, *Salmonella* (*S.)* Typhimurium and *S.* Enterica, shigatoxin-producing *E. coli*, and *Listeria (L.) monocytogenes* [56]. These pathogens have been detected at various stages along the meat production chain, including pre-harvest (farm animals), harvest (raw meats), and post-harvest (RTE meats) stage [49].

These pathogens lead to distinct foodborne illness manifestations: *C. jejuni* causes campylobacteriosis, a gastrointestinal disease characterized by diarrhea, fever and abdominal cramps; *S.* Enterica infection results in fever, headache, nausea, vomiting, abdominal pain, and diarrhea; toxin producing *E.coli* O157 causes mild bloody diarrhea or even severe hemorrhagic colitis, and *L. monocytogenes* can lead to serious conditions such as meningitis, newborn septicemia, encephalomyelitis, and death particularly in the elderly, pregnant women or newborns [57].

Campylobacter spp. infections, predominantly associated with poultry meat, rank as the most commonly reported foodborne illness worldwide [14]. The European Food Safety Authority (EFSA) reported campylobacteriosis as the most frequently documented foodborne zoonosis in the EU, with 220.682 cases reported across 18 constituent member states in 2019 [58]. Notably, poultry meat consumption is associated with the majority of human campylobacteriosis cases (50-80%) [59].

In poultry farms, *Campylobacter* spp. primarily spread within a flock through the fecal-oral route [60]. Once introduced to a flock, *Campylobacter* spp. spreads rapidly, reaching high levels (between 10^6 and 10^8 colony forming units (CFU) per g) in the intestinal tracts of most birds within a few days [61]. *Campylobacter* spp. can survive outside hosts, contributing to contamination sources such as farm equipment, transportation vehicles, farmworkers, drinking water, feed, litter, and air [62].

As chicken intensities provide ideal conditions for *Campylobacter* spp. growth, it is crucial to prevent spreading of this pathogen on poultry farms before it spreads through the entire meat production chain [63]. Drinking water was identified as a potential risk factor contributing to *Campylobacter* spp. colonization in poultry during the fattening period, posing a risk of flock infection [58,64]. Thus, it was hypothesized that the microbial contamination level in poultry drinking waterlines correlates with presence of *Campylobacter* spp. Consequently, this thesis focused on evaluating the contamination level in poultry drinking water before and after waterline sanitation practices on Austrian poultry farms, with a specific emphasis on identifying *Campylobacter* spp.

1.5 Process-related microbiota in pork meat processing environment

Microbial proliferation in meat processing environment is a critical factor leading to the meat spoilage, ultimately resulting in the degradation of meat quality and rendering it unsuitable for human consumption [28]. Among the bacteria causing spoilage under refrigerated conditions, Gram-negative bacteria like pseudomonads, *Enterobacteriaceae, Shewanella putrefaciens*, along with several Gram-positive bacteria some of them being lactic acid bacteria (LAB), *Brochothrix (B.) thermosphacta*, and clostridia dominate under these conditions [42]. Focusing on pork meat processing environment, prevalent process-related bacteria associated with meat spoilage include

B. thermosphacta, Carnobacterium spp., *Enterobacteriaceae*, *Latilactobacillus* spp., *Leuconostoc* spp., *Pseudomonas* spp., and *Weissella* spp. [39,42,65].

Within pork meat processing environment LAB stand out as extensively adapted microorganisms, significantly influencing the quality and safety of pork products [37]. The LAB are a group of microorganisms that have a complex role in fresh meat and cooked meat products. LAB strains can produce undesirable compounds that degrade the meat quality, while others act as protective agents, inhibiting pathogenic bacteria and spoilage-causing microorganisms [42].

Modified atmosphere packaging (MAP) and vacuum packaging are widely used preservation methods that minimize changes in fresh meat and RTE pork meat products, meeting consumer demand for additive-free foods with extended shelf-life [66]. These techniques, often combined with low-temperature storage, select for psychrotrophic, anaerobic, and facultative anaerobic microbes, such as LAB. LAB dominate in these packaged chilled pork products until the end of their shelf-life due to their adaptability to cold temperatures, CO_2 resistance, and low oxygen tolerance [67].

Consequently, fresh and RTE pork meat products harbor various LAB genera and species involved in meat spoilage and preservation, such as *Leuconostoc*, *Latilactobacillus*, *Carnobacterium*, *Lactococcus*, and *Enterococcus* are the main genera associated with pork meat spoilage [42]. Simultaneously, many LAB species have received Generally Recognized as Safe (GRAS) or Qualified Presumption of Safety (QPS) status by regulatory authorities and are used as starter cultures in meat production [68].

Recent scientific attention has shifted towards strictly psychrotrophic LAB species found in vacuum-packed RTE pork products [69,70]. These bacteria constitute the primary microbiota in these products throughout their shelf-life, yet their growth patterns remain largely uncharacterized. Additionally, previous studies on LAB associated with meat spoilage often overlooked variations at the strain level. In the

present study it was hypothesized that LAB contribute to fluctuations observed in vacuum-packed cooked ham and that these LAB originate from both raw meat and the processing environment of cooked ham. Consequently, this thesis aimed to investigate the growth patterns of the predominant LAB species at the strain level during the refrigerated storage of vacuum-packed cooked ham. Moreover, the research focused on tracing these bacteria within the pork processing environment, offering crucial insights into potential routes of product cross-contamination and re-contamination. This investigation ultimately aimed to enhance and equip meat production companies with improved strategies for managing and controlling these bacteria within their operational environments.

1.6 Microbiological assessment of meat safety and quality

Effective food safety management relies on solid evidence that hazards are well controlled, and the interplay between initial microbial levels, reduction, recontamination, and growth results in an appropriate final level or prevalence of the hazard (**Figure 1**) [71]. This evidence is obtained through validation, which may involve sampling to gather data on initial levels and prevalence of microbiological contaminants in animal production farms, raw materials, and the processing environment. The validation demonstrates that a process is under control, and this can be further verified through finished product testing at the industry level and epidemiology. However, the absence of a microbial hazard in finished products or lack of evidence for an epidemiological link does not guarantee that a process and food product safety are fully under control [71]. Conversely, non-compliance with finished product standards or the presence of a strong epidemiological link may indicate a lack of control in the process.

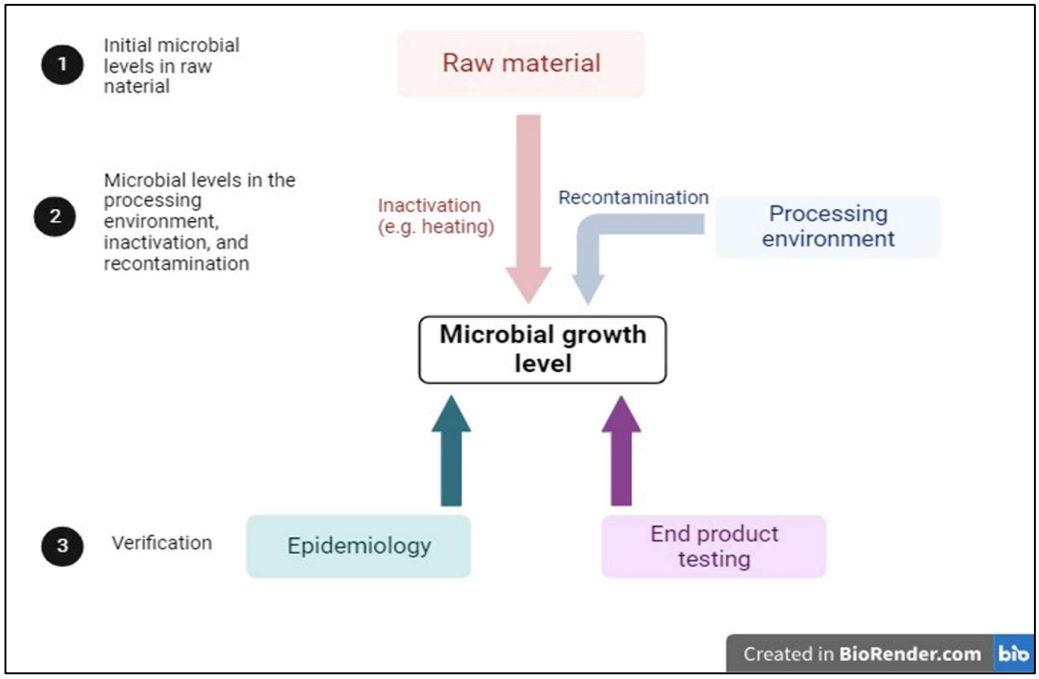

Figure 1: Schematic overview of effective food safety management in meat processing facility.

The assurance of food quality and safety is shifting towards proactive approach, emphasizing prevention and control measures rather than relying solely on end product testing [71]. Control activities focus on reducing or preventing food safety hazards and typically involve product and process controls [72]. Preventive measures, such as cleaning, sanitation, temperature control, and worker hygiene, are implemented to prevent contamination and microbial growth. Production interventions may involve reducing or eliminating specific contaminants through heat treatments.

On the other hand, assurance activities in a food safety management system aim to provide evidence that products and processes meet established specifications. Examples of assurance activities include sampling, validation, verification, and documentation [72]. Detecting foodborne pathogenic and spoilage bacteria in their early growth stages is vital for mitigating the occurrence of foodborne outbreaks and minimizing significant food and economic losses due to microbial spoilage [5]. Food businesses are prioritizing the design and implementation of food safety management systems to ensure the safety

of their products. Therefore, the implementation of stringent procedures and regulations, such as GMP and HACCP, is not only a legal requirement but also a fundamental measure aimed at preventing foodborne outbreaks [73].

The most commonly used approaches in bacteriological analysis in food microbiology are aiming at are detection and enumeration of either total microflora or particular species according to International Organization for Standardization (ISO) reference methods and EC regulation 2073/2005 [26]. Food microbiology employs diverse microbiological testing methods, including culture-based methods, immunological assays, nucleic acid-based methods (polymerase chain reactions (PCR)), and next generation sequencing (NGS) methods [74–76].

The conventional approach for assessing the composition of food-associated microbiota relies on culture-based methods. It involves the isolation and cultivation of microorganisms before their identification and typing. In food microbiology, culture-based methods remain the most direct approach to study the presence of microorganisms, thus serving as the gold standards for most of the microorganisms involved in food microbiology considering that national and international regulatory agencies must use tests that are legally acknowledged [77]. Therefore, culture-based methods still remain highly significant for routine quality and safety management in food production companies. As the primary focus of the thesis was on meat safety and quality, in line with national and international microbial quality guidelines, the investigation relied on culture-based ISO methods.

1.6.1 Culture-based methods in food microbiology

Hygiene criteria are established for aerobic mesophilic count (AMC) and *Enterobacteriaceae* (EB) [78]. Food safety criterion for pathogens such as *Salmonella* spp. and *L. monocytogenes* are established in EC regulation 2073/2005 [79]. Additionally, *Campylobacter* spp. are designated as process hygiene criteria in

Commission Regulation (EU) 2017/1495 of 23 August 2017, which amends Regulation (EC) No 2073/2005 concerning *Campylobacter* spp. in broiler carcasses [80]. The AMC and LAB serve as an indicator of overall contamination, shelf-life, or spoilage, without posing a direct health risk [81]. The EB and *Pseudomonadaceae* (PS) on the other hand, are used to assess the hygiene status of the food production processes [82–84].

Standardized methods, such as ISO methods, are recognized as the official reference analytical methods for quality control [85]. Developed through expert knowledge and international consensus, these methods, known as the "gold standard" in food diagnostics, are widely accepted and employed by many laboratories, especially regulatory agencies, to ensure compliance [78,85].

Culture-based methods have been developed for pathogens and spoilage organisms, focusing on the presence or absence and/or enumeration of these microorganisms, to meet legal and regulatory food safety standards [86]. Despite the availability of modern methods, culture-based methods remain the reference for detecting food borne pathogens [74]. They utilize selective broth and agar media to multiply target organisms until they reach detectable levels, visible by turbidity, color change, or colony formation. Culture-based detection of four major pathogens, *Salmonella* spp., *Campylobacte*r spp., *L. monocytogenes* and *E. coli* O157, being the subject of surveillance programs and intensive testing, normally employ enrichment-plating techniques, as these pathogens in foods may be sub-lethally injured [87]. After enrichment the target organisms is isolated on a selective differential agar medium. Culture-based methods are most effective when the growth requirements of target microorganisms are known, as culture media can be used to selectively enrich, isolate, and differentiate them. For liquid foods, the quantification limit is approximately 4 CFU/ml, while for solid foods, it is approximately 40 CFU/gram (CFU/g) [85]. Additional tests such as colony characteristics, Gram staining, biochemical characterization, and serological or nucleic acid-based methods can be employed for definitive identification [88]. Standard ISO method for detection of *Campylobacter* spp., *E. coli* O157, *L. monocytogenes*, and

Salmonella spp. in food and feed include ISO 10272-1:2017, ISO 16654:2001, ISO 11290-1:2017, and ISO 6579:2017, respectively. The ISO standards for AMC, LAB and EB in food and food processing facilities include ISO 4833-2:2013, ISO 15214:1998, and ISO 21528-2:2017, respectively.

1.6.2 Molecular-based identification methods

Some bacteria are challenging to identify using common phenotypic identification schemes outside the reference laboratories. Variability in phenotypic characteristics, such as morphology, carbohydrate utilization profiles, and enzyme patterns, among strains of the same species can result in atypical characteristics for identification [89,90]. Molecular identification techniques like PCR is relevant for detecting bacteria in food [85]. For pathogen detection in food, ISO accredited PCR methods recommend a prior enrichment step of 6-24 hours before performing PCR, as only a small volume of DNA (1 ml) is processed [91,92]. Multiplex PCR, which amplifies multiple loci simultaneously, is necessary for rapid detection of multiple microorganisms in a single reaction [93]. This technique combines several specific primer sets into a single PCR assay. In this thesis, colony multiplex PCR was employed to simultaneously identify the *hipO* gene (hippuricase) from *Campylobacter (C.) jejuni subsp. jejuni*, the *glyA* gene (serine hydroxymethyltransferase) from *C. coli, C. lari,* and *C. upsaliensis*, and the *sapB2* gene (surface layer protein) from *C. fetus* subsp. *fetus* [94]. In addition to phenotypic characterization of the isolates, we employed partial sequencing of the 16S rRNA gene for taxonomic classification and identification of the bacterial isolates in this thesis.

16S rRNA gene sequencing provides genus and species identification for isolates that do not match recognized biochemical profiles of commercial identification systems [95]. While initially designed for identification of uncultured microorganisms, 16S rRNA gene sequencing has become increasingly gained practical applicability in the routine investigation of cultured bacteria [89]. The technique is now commercially

available, and large public-domain databases exist for bacterial phylogeny based on 16S rDNA. It has been extensively used for bacterial identification, including uncultured microorganisms, unique or unusual isolates, and phenotypically identified isolates. The use of 16S rRNA gene sequencing for organism identification has been documented in various case reports and studies focusing on specific bacterial phyla or genera, such as *Mycobacterium, Actinomyces*, aerobic Gram-negative bacilli, coryneform bacteria, and aerobic Gram-positive rods [96–100]. However, 16S rRNA gene sequencing has limitations in species-level phylogenetic and discriminatory power, especially for some genera like *Bacillus, Enterobacteriaceae* (in particular, *Enterobacter* and *Pantoea*), rapid-growing mycobacteria, the *Acinetobacter (A.) baumannii-A. calcoaceticus complex, Achromobacter, Stenotrophomonas* spp., and *Actinomyces* spp. [95].

In the absence of a universal definition for species identification using 16S rRNA gene sequencing, authors vary widely in their criteria. It is recommended that microbial identifications using 16S rRNA distance scores greater than 1% are inadequate for diagnostic or public health reference laboratories. To establish an accurate isolate identification, a species-level similarity of 99% and a genus level similarity of ≥97% are recommended, which was considered in the present thesis [95].

1.6.3 Genotypic-based identification methods

By identifying and tracing recontamination sources using molecular typing methods, a better understanding of the specific micro-flora in food processing environment can be achieved [101]. In this thesis, molecular typing method was employed to detect variations among bacterial isolates within a species, allowing for the characterization of specific subtypes. This facilitated the identification of subpopulations with unique traits. This assisted in identifying associations between the end product, raw material, and the processing environment.

Standard subtyping methods along with phenotypic identification, play a crucial role in distinguishing bacterial strains. Genotyping, which involves DNA-based analysis of chromosomal or extrachromosomal genetic material, offers a significant advantage in its ability to discriminate closely related species [102]. The choice of an appropriate molecular typing method depends on the specific problem and epidemiological context. The method must be highly discriminatory, cost-effective, reproducible, easy to perform and interpret. Stability over time is important for continuous surveillance to enable effective infection control measures [103].

The pulsed-field gel electrophoresis (PFGE) molecular typing method was used to characterize bacterial isolates identified during storage of cooked am RTE product and in cooked ham processing environment. It involves analyzing chromosomal DNA restriction patterns by embedding organisms in agarose, lysing them, and digesting the DNA with restriction endonucleases [104]. The PFGE was employed as a tool to further characterize LAB at the strain level due to its high discriminatory power, epidemiological concordance, and has been successfully used in large-scale investigations [105].

2 AIMS OF THE STUDY

Studying the microbial quality and safety of meat is essential for human and animal health, ensuring food safety, and regulatory compliance across the entire meat production chain. The current research work focused on two main areas vital to meat safety and quality, including poultry primary production and production of RTE cooked ham pork product. The investigations involved application of culture-based detection methods, such as AMC, LAB, EB, and PS bacteria, to assess overall contamination during meat production.

Firstly, a comprehensive evaluation was conducted on poultry farms employing different waterline sanitation practices. The aim was to assess the implications of these practices on overall drinking water quality used for poultry production. Additionally, the presence of *Campylobacter* spp., a human pathogen, in poultry drinking water was evaluated, followed by the identification of antibiotic resistance patterns in most commonly isolated bacteria found in poultry drinking water.

Secondly, the investigation focused around assessing the microbial stability of cooked ham, an RTE pork product, throughout its shelf-life. In addition, the study traced the most commonly isolated bacteria during the cooked ham shelf-life back to raw meat and processing environment. Furthermore, metabolic traits were examined in LAB isolates obtained from raw meat, the processing environment, and cooked ham.

It was hypothesized that specific microorganisms could impact the presence and abundance of pathogenic and spoilage organisms within the meat processing environment, influencing meat product safety and quality. In conclusion, the aim of the thesis was to study microbial diversity, identify transmission routes within the meat production facility, and to propose novel strategies to mitigate pathogens and spoilage bacteria within poultry and pork meat production chain.

3 ORIGINAL MANUSCRIPTS

3.1 Assessment of microbial quality in poultry drinking water on farms in Austria

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Assessment of microbial quality in poultry drinking water on farms in Austria

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The quality of poultry drinking water has a significant effect on broiler health and performance. This study conducted an analysis of aerobic mesophilic counts (AMC), *Enterobacteriaceae* (EB), *Pseudomonadaceae* (PS), and screened for the presence of *Campylobacter* spp. in water samples collected from a total of 14 farms in Austria, with either a public or private water source. The efficacy of two water line treatment methods was evaluated: a chemical treatment of the water lines with 4.0 ppm ClO_2 (T1) and a combined chemical (4.0 ppm active ClO_2 and 3.0% peracetic acid) and mechanical treatment (purging of the water lines with a high-pressure air pump; T2). However, both the T1 and T2 treatments failed to reduce the AMC counts below the maximum acceptable microbial limit of 4.0 log_{10} CFU/ml in water samples. In addition, no significant reduction in EB and PS counts was observed in water samples after either T1 or T2 water line treatment. The water samples showed a high level of microbial diversity with 18 to 26 different genera. The genus *Pseudomonas* was most frequently isolated across all poultry farms, while *Campylobacter jejuni* was identified in a single sample collected before water line treatment. Isolate analysis revealed the presence of opportunistic pathogens in water samples both before (T1 43.1%, T2 30.9%) and after (T1 36.3%, T2 33.3%) water line treatment. Opportunistic pathogens belonging to genera including *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp., were most frequently isolated from poultry drinking water. These isolates exhibited multidrug resistance and resistance phenotypes to antimicrobials commonly used in Austrian poultry farms. The findings of this study emphasize the potential risk of exposure to opportunistic pathogens for poultry and personnel, underscoring the importance of efficient water line management.

KEYWORDS

water line treatment, opportunistic pathogens, poultry health, *Pseudomonas*, antimicrobial susceptibility

1 Introduction

Poultry is one of the main sources of meat production worldwide (1). In 2020, more than 97 million chickens were processed in Austria, representing 124.000 tons of processed poultry meat (2). Drinking water is a vital nutrient for commercial poultry and has a significant impact on poultry health, liveweight, feed conversion ratios, and overall performance (3, 4). The water consumption of poultry is approximately twice the amount of feed intake (5). Poultry health and water intake are directly influenced by microbial water quality (4, 6, 7).

In Europe, the water quality standards for poultry drinking water have been adapted from water quality regulations intended for human drinking water consumption (8), EC Directive 98/83/EC (Drinking Water Directive [DWD] 9). According to the Austrian Poultry Hygiene Regulation (10) drinking water used for poultry production must not exceed a total aerobic mesophilic count (AMC) of 2.0 $\log_{10}$ and 1.3 $\log_{10}$ colony forming units (CFU/ml) at 22° and 37°C, respectively. Currently, there is no legal requirement to examine microbial contamination inside the drinking water lines (11). Hence, maintenance of water line hygiene is primarily the responsibility of the poultry producer, and it is typically conducted between the production cycles (12). The standard water line practices involve mechanical cleaning by flushing the water lines, followed by oxidative disinfection, primarily using chlorination or acidifiers (7, 12–14).

While water line treatment is a crucial component of an effective biosecurity program, its effectiveness does not ensure the complete elimination of the microorganisms within the water lines (15–17). *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. have been detected in poultry drinking water (7, 18). Elevated temperatures and low water flow rates in enclosed water line systems have been found to adversely affect water quality, as indicated by previous studies (4, 12). These conditions are favorable for the accumulation of dissolved organic substances, minerals, and solid particles, which facilitate growth and promote the formation of biofilms. Among biofilm-forming bacteria, primarily *Pseudomonas* and *Stenotrophomonas* are responsible for biofilm formation on surfaces of poultry drinking lines (12). Biofilms may provide a favorable surface for attachment of opportunistic pathogens (OP), such as such as *Acinetobacter*, *Aeromonas, Citrobacter, Enterobacter,* and *Klebsiella* whose members are natural inhabitants of plumbing systems and adapted to survival in drinking water (19). Although these bacteria are generally not pathogenic, some have the potential to cause infections in susceptible poultry and farm workers (20). Hence, the detachment of pathogen and OP rich biofilms and their contamination of the water system present a significant risk for waterborne transmission of these bacteria, posing a potential threat to both poultry and human health. Moreover, the administration of medication to poultry through drinking water, which is a preferred route, has been linked to presence of multidrug-resistant (MDR) bacteria (21, 22).

Microbial water quality is frequently evaluated at its source, but assessments at the end of the drinking lines are infrequent, despite the potential for substantial variations in microbial quality between the source and endpoint (12). Thus, the objective of this study was to evaluate the microbial quality of water samples collected at the end of a production cycle of five to six weeks and shortly before restocking for the subsequent production cycle, following the water line treatment. Previous studies have demonstrated the presence of pathogens such as *Campylobacter* spp. in poultry water on farms with private water supplies compared to those with a public supply (23, 24). This highlights the critical role of poultry drinking water as a potential source of *Campylobacter* spp. infection on the farm (25, 26). The presence of *Campylobacter* spp. in drinking water on poultry farms may indicate lapses in biosecurity, contaminated water source, ineffective and/or incorrectly applied water line cleaning procedures (11, 18). Therefore, one of our objectives was to assess the microbial quality of poultry drinking water in farms with either public or private water supply. We applied ISObased reference methods to assess bacterial load and presence of *Campylobacter* spp. in poultry drinking water, followed by partial 16S rRNA sequencing of bacterial isolates. Antibiotic susceptibility patterns of commonly isolated OP were then determined.

2 Materials and methods

2.1 Water line treatment and sample collection

Twenty-eight poultry farms producing broilers for local slaughterhouses in Austria voluntarily participated in the study between May 2019 and August 2020, some of which had private ($n = 11$) and others public ($n = 17$) water supplies. The fattening period at the participating poultry farms in Austria was five to six weeks. The poultry farms were divided into two distinct groups based on whether the farms employed solely chemical (T1) or a combination of chemical and mechanical (T2) water line treatment methods. An overview of the poultry farms included in the study is presented in Figure 1. Cleaning and water line treatment at the poultry farms was performed by the farmer. Since the participation of poultry farms in the study was voluntary, poultry farms 6, 8, 9, 12, and 13 withdrew their participation after T1 and were substituted by the poultry farms 15–19 during T2. The study was conducted in collaboration with a private laboratory (HYGIENICUM GmbH, Graz, Austria), which provided training on the water line cleaning procedures to be implemented at the poultry farms to the participating farmers.

During T1 water line treatment, water lines were drained and filled with a commercially-available solution of which the main disinfecting component contained 4.0 ppm active chlorine dioxide (ClO_2) solution (Calgonit CD-K1/K2, Calvatis GmbH, Ladenburg, Germany). The commercial solution was retained in the water lines for 24 h. Measurements of free ClO_2 inside the waterlines were not obtained. Subsequently, the water lines were washed with the supply water by continuous flushing for 10 min. Under normal operating conditions. The T2 water line was performed by continuous pumping of acidic cleaner containing 3.0% peroxyacetic acid (PAA) and hydrogen peroxide (Calgonit DS 625, Calvatis GmbH, Ladenburg, Germany) continuously for 30 min using highpressure air pump. The water lines were then washed with the supply water and purged using a high-pressure air pump until no inorganic and organic debris were visible in the water. Subsequently, the water line disinfection was performed using a commercial disinfection solution containing 4.0 ppm active ClO_2 solution (Calgonit CD-K1/K2) which was retained in the water lines for 24 h. Subsequently, the water lines were washed with supply water by flushing for 10 min. Under normal operating conditions.

Water samples were collected by employees from the private laboratory, samples were taken from the end nipple of the drinking

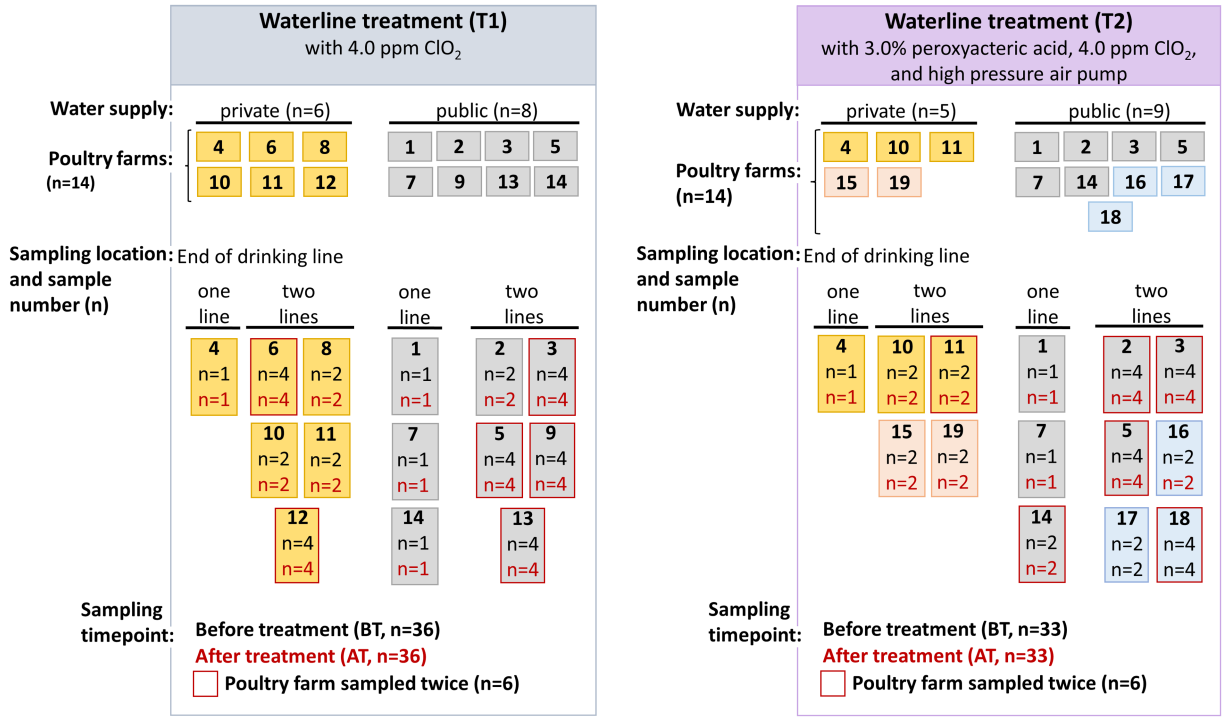

FIGURE 1
An overview of the sampling conducted in fourteen poultry farms during chemical (T1) and combined chemical with mechanical (T2) water line treatment. Poultry farms 6, 8, 9, 12, and 13 withdrew their participation after T1 and were substituted by the poultry farms 15 and 19 (indicated by pink color) with private water supply, and 16–18 (indicated blue color) with public water supply during T2 water line sampling.

water line inside the vacant poultry house (HYGIENICUM GmbH, Graz, Austria). One water line was sampled at four and five poultry farms, while two water lines (line 1 and 2) were sampled at ten and nine poultry farms during T1 and T2 water line treatments (Figure 1). Two sampling timepoints were chosen, namely before treatment (BT) at the end of fattening period of 5–6 weeks, and after the water line treatment (AT) before restocking of the subsequent production cycle. As shown in Figure 1 and Supplementary Table S1, in six poultry farms during the T1 and T2 water line treatment, water samples were collected at two different sampling intervals, while other poultry farms were sampled only once. Additionally, at some poultry farms from some water lines the duplicate samples were collected, while from other poultry farms only a single sample was collected. Therefore, in total 36 (T1) and 33 (T2) BT and corresponding AT samples were collected for the microbial analysis in the present study. The water samples were collected in sterile 500 mL bottles by the private laboratory and immediately transported to the laboratory at 4°C for microbial analysis.

2.2 Sample processing and microbial analysis

Prior to analysis, 500 mL of water samples were centrifuged at 8000 rpm for 30 min at 4°C (Thermo Scientific, Sorvall Lynx 4000 centrifuge). All but 10 mL of the supernatant was discarded, the remainder was then resuspended using a serological 10 mL pipette (Greiner Bio One, Frickenhausen, Germany) and vortexed for 30 s.

Campylobacter selective enrichment and isolation were performed according to the ISO 10272-1:2006 standard for the detection of *Campylobacter* spp. in foodstuff (27). Five milliliters of the supernatant were transferred to 45 mL of Bolton broth (Thermo Fisher Scientific Ltd., Hampshire, United Kingdom) supplemented with 5% hemolyzed horse blood (Oxoid Ltd., Hampshire, United Kingdom). The Bolton broth enrichment was incubated for up to 48 h at 42°C under microaerobic conditions (10% CO_2, 3% O_2, 87% N_2). After incubation modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid Ltd) was inoculated by fractionated loop inoculation (10 µL) and incubated at 42°C for 48 h under microaerobic conditions. Quantification of aerobic mesophilic count (AMC), *Enterobacteriaceae* (EB), and *Pseudomonadaceae* (PS) counts was carried out according to ISO reference methods (28, 29). For enumeration of AMC, EB, and PS, 5 mL of the re-suspended supernatant was transferred to 45 mL buffered peptone water (BPW) (Biokar Solabia diagnostics, Pantin Cedex, France). Subsequently, serial ten-fold dilutions were prepared up to dilution 10^{-5} in BPW (Biokar Solabia diagnostics, Pantin Cedex, France). The AMC were enumerated on trypto-caseine soy agar with 0.6% yeast extract (TSAYE) (Biokar Solabia diagnostics), while EB and PS were enumerated on red bile glucose agar (VRBG) (Merck KGaA, Darmstadt, Germany). Each dilution step (100 µL) was plated on selective agar media for the enumeration of AMC, EB, and PS counts. For dilution 10^{-1} the volume of 1 mL was divided (333 µL) on three agar plates per selective medium. Agar plates were incubated at 30°C (AMC) and 37°C (EB, PS) aerobically for up to 48 h. The EB and PS counts on VRGB agar were differentiated by their ability to ferment

glucose, leading to pink colonies with or without precipitation and pale colonies for PS. Presumptive EB and PS isolates were confirmed using oxidase reaction (BioMerieux, Marcy l'Etoile, France). The minimum and maximum limits for the determination of the AMC, EB, and PS in the samples ranged between 10 and 300 CFU.

Microbial quality of water samples before (BT) and after (AT) sanitation were categorized according to AMC, EB, and PS load in two contamination levels, <4.0 $\log_{10}$ CFU/ml and ≥4.0 $\log_{10}$ CFU/ml based on existing studies (4, 7, 12).

2.3 Isolation and identification of bacterial and *Campylobacter* spp. isolates

The predominant bacterial colony morphologies were collected from each water sample for further confirmation. Specifically, 1–5 colonies were selected from TSAYE ($n = 224$), VRBG ($n = 206$) and mCCDA agar ($n = 41$) and then subcultured on the respective medium. The isolate list is provided in the Supplementary Table S1. The purified colonies, comprising isolates from T1 BT samples ($n = 123$), T1 AT samples ($n = 113$), T2 BT samples ($n = 139$), T2 AT samples ($n = 96$) were stored at – 80°C in brain heart infusion broth (Biokar Solabia diagnostics) supplemented with 25% (v/v) glycerol (Merck KgaA).

For DNA extraction of *Campylobacter* spp. isolates 10 µL loop of bacterial material was resuspended in 100 µL of 0.1 M Tris–HCl buffer pH 7 (Sigma Aldrich, St. Louis, MO, United States) and mixed with 400 µL Chelex® 100-Resin (BioRad, Hercules, CA, United States) (30). The bacterial Chelex® 100Resin suspension was heated at 100°C for 10 min on a block heater (Thermo Fischer Scientific Inc.), followed by short centrifugation step at 15,000 ×*g* (Eppendorf Centrifuge 5,425) for 5 s. The supernatant (100 µL) was transferred to a maximum recovery tube (Corning Incorporated Life Sciences, Reynosa, Mexico) and stored at −20°C until analysis. *Campylobacter* spp. were identified using multiplex PCR targeting genes including the conserved genus-specific 23S rRNA gene, the *Campylobacter jejuni* hippuricase gene (*hipO*) and the *Campylobacter coli* serine hydroxymethyltransferase (*glyA*) gene, as previously described (31). Briefly, a single reaction mixture (20 µL) contained diethylpyrocarbonate (DEPC) treated water (Sigma Aldrich), 1× PCR buffer, 2 mM $MgCl_2$, 500 nm *hipO* forward and reverse primer, 1,000 *glyA* forward and reverse primer, 200 nm 23S forward and reverse primer, 200 µM dNTP mix, 1.5 U of Platinum Taq DNA polymerase (Platinum™ Taq DNA Polymerase, DNAfree, Thermo Fisher Scientific Inc., Waltham, MA, United States), and 5 µL template genomic DNA. The amplification was performed in T100™ Thermal Cycler (Bio-Rad, Hercules, CA, United States). The PCR cycling conditions included initial denaturation at 94°C for 2 min, 30 cycles of denaturation (94°C for 30 s), primer annealing (59°C for 30 s), elongation (72°C for 30 s) and final elongation (72°C for 7 min). The gel electrophoresis of PCRamplicons was performed in a 1.5% agarose gel containing 0.5× TrisBorateEDTA (TBE) buffer (Sigma Aldrich, St. Louis, MO, United States) and 3.5 µL peqGREEN DNA gel stain (VWR International, Radnor, United States), at 120 V for 30 min. The DNA standard Thermo Scientific™ GeneRuler™ 100 bp (Thermo Fisher Scientific Inc., Waltham, United States) was applied for fragment length comparison. We utilized the following control isolates for the DNA extraction and multiplex PCR: *C. jejuni* strain DSM 4688 and *C. coli* strain DSM 4689, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

For DNA extraction of isolates from TSAYE and VRBG, bacterial cells were lysed by boiling the suspension. A 10 µL loop of bacterial material was re-suspended in 100 µL 0.1 M Tris–HCl pH 7 buffer (Sigma Aldrich, St. Louis, MO, United States), briefly vortexed and heated at 100°C for 15 min (Thermo Scientific™ block heater, Thermo Fischer Scientific Inc.). The suspension was then centrifuged for 5 s at 15,000 ×*g* (Eppendorf Centrifuge 5,425, Hamburg, Germany) and the supernatant (70 µL) was transferred into maximum recovery tubes (Corning Incorporated Life Sciences, Reynosa, Mexico) and stored at −20°C until analysis. For identification of bacteria isolates ($n = 471$) the partial amplification of 16S rRNA gene was performed following the methods of (32, 33), using universal primer pairs 616F (5'AGAGTTTGATYMTGGCTC3') and 1492R (5'GGYTACCT TGTTACGACTT3') (both Microsynth AG, Blagach, Switzerland). A single PCR reaction (45 µL) contained 1× PCR buffer, 2 mM $MgCl_2$, 200 nM forward and reverse primer, 250 µM dNTP mix, 2 U of Platinum Taq DNA polymerase (Platinum™ Taq DNA Polymerase, DNAfree, Thermo Fisher Scientific Inc.) and 5 µL template genomic DNA. The DNA amplification was performed in T100™ Thermal Cycler (BioRad, Hercules, CA, United States). The PCR cycling conditions included initial denaturation at 95°C for 5 min, 35 cycles of denaturation (94°C for 30 s), primer annealing (52°C for 30 s), elongation (72°C for 60 s) and final elongation (72°C for 7 min). Subsequently, the PCR amplicons were sent for purification and sanger sequencing to LGC Genomics (LGC Genomics GmbH, Berlin, Germany). The gel electrophoresis of PCR-amplicons was performed in a 1.5% agarose gel containing 0.5× TrisBorateEDTA (TBE) buffer (Sigma Aldrich, St. Louis, MO, United States) and 3.5 µL peqGREEN DNA gel stain (VWR International, Radnor, United States), at 120 V for 30 min. The DNA standard Thermo Scientific™ GeneRuler™ 100 bp (Thermo Fisher Scientific Inc., Waltham, United States) was applied for fragment length comparison. The PCR amplicons were sequenced using a 1492R (5'GGYTACCTTGTTACGACTT3') primer. The nucleotide sequences were qualityevaluated by using Finch TV 1.4.0 (34) and MEGA X (35). The bacterial nucleotide Basic Local Alignment Search Tool (BLAST) algorithm from the National Centre for Biotechnology Information (NCBI)[1] was used for taxonomy assignment. Sequences were assigned to genus or species level according to best matches and highest similarities (1,040 to 1,120 bp fragment length, similarity cutoff ≥97.0%). The partial rRNA gene sequence data from the isolates were deposited in the GenBank database under accession numbers MZ642358 to MZ643011.[2] Subsequent identification of opportunistic pathogens among identified isolates was performed using the bacterial metadata base BacDive (36) and List of Prokaryotic names with Standing in Nomenclature (LPSN) (37).

2.4 Antimicrobial susceptibility testing

Opportunistic pathogens with clinical relevance isolated from water samples during T1 and T2 water line treatment were subjected

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

2 https://www.ncbi.nlm.nih.gov/genbank

to antimicrobial susceptibility testing (AST). The set of isolates included most frequently isolated OP, such as *Pseudomonas* spp. ($n = 17$), *Ochrobactrum* spp. ($n = 4$), *Stenotrophomonas* spp. ($n = 3$), and human relevant opportunistic pathogens including *Citrobacter* spp. ($n = 2$), *Enterobacter* spp. ($n = 2$), *Klebsiella* spp. ($n = 1$), and *Aeromonas* spp. ($n = 1$).

AST was performed for a total of 30 bacterial isolates using Sensititre™ Avian AVIAN1F Vet AST Plate (ThermoFischer Scientific Inc., Waltham, MA, United States), according to the manufacturer's instructions. Briefly, single colonies were picked from fresh cultures grown on TSAYE for 24 h at 30°C, suspended in in sterile water to an optical density of a 0.5 McFarland standard (~ 10^8 CFU/mL). 50 µl volumes of the bacterial suspension were transferred to wells containing different concentrations of lyophilized antimicrobials. Plates were sealed and incubated at 30°C for 24 to 48 h, after which minimum inhibitory concentrations (MIC) were read visually and defined as the lowest concentration of a given antibiotic at which no growth of the test organism was observed. *E. coli* strain ATCC 25922 was used as the internal quality control isolate. The minimum inhibitory concentration (MIC) breakpoints and definitions for multi-drug resistance (MDR; resistance to two or more antibiotic classes) (38) were determined following the standards provided by the Clinical and Laboratory Standards Institute (CLSI) manuals (39–41).

2.5 Data analysis

A descriptive analysis was carried out (mean, median, and standard deviation) for AMC, EB, and PS counts. The normal distribution of each data set (T1 and T2) was investigated using the Shapiro–Wilks test. Due to nonnormal distribution of data, the median values of AMC, EB, and PS counts were calculated. The Wilcoxon–Mann–Whitney rank sum test performed as a twosided test was applied to identify whether there was a significant difference between median AMC, EB and PS counts of BT and AT samples. Median AMC, EB, and PS counts in AT samples were compared for different water supplies (public vs. private), water line treatments (T1 vs. T2), following $\log_{10}$ transformation, using Wilcoxon–Mann–Whitney rank sum test. Values of $p < 0.05$ were considered as statistically significant. Statistical analyses were carried out using the R software package for statistical computing.[3]

3 Results

3.1 Aerobic mesophilic count, *Enterobacteriaceae*, and *Pseudomonadaceae* count in poultry drinking water

Ninety-nine BT samples and their corresponding AT water samples were microbiologically assessed, with a maximum acceptable microbial limit of 4.0 $\log_{10}$ CFU/ml for AMC, EB, and PS counts (Table 1). Due to non-normal distribution of the data, we used the Wilcoxon–Mann–Whitney twosided rank sum test to assess the median values for AMC, EB, and PS counts. No significant differences ($p \geq 0.05$) were observed between the median AMC, EB, and PS counts of the BT and AT samples after T1 water line treatment (Table 1). Furthermore, we did not observe any significant difference between median AMC, EB, and PS counts in poultry farms with private and public water supply. Among the water samples, the highest median AMC counts were observed in BT (5.9 ± 1.02 $\log_{10}$ CFU/ml, median ± MAD; MAD: median absolute deviation) and AT (6.0 ± 1.17 $\log_{10}$ CFU/ml) samples. Higher median AMC counts in BT and AT samples were observed in poultry farms with a private well than those with a public water supply (Table 1). The lowest median counts were observed for EB in both BT (3.6 ± 2.13 $\log_{10}$ CFU/ml) and AT (2.3 ± 1.52 $\log_{10}$ CFU/ml) samples. In AT samples higher median EB counts were observed in poultry farms with public water supply. The PS resulted in the second highest median counts, which remained unchanged in BT (4.7 ± 1.44 $\log_{10}$ CFU/ml) and AT (4.7 ± 2.48 $\log_{10}$ CFU/ml) samples. Higher median PS counts were detected in both BT and AT samples in poultry farms with public water supply.

After T1 water line treatment, high (>4.0 $\log_{10}$ CFU/ml) AMC, EB, and PS counts from BT samples decreased below the maximum acceptable microbial limit in 8/36, 7/36, and 9/36 AT samples, respectively (Supplementary Table S2). The AMC, EB, and PS below the microbial limit were observed in 1/36, 18/36, and 7/36 BT and AT samples, respectively. The AMC, EB, and PS counts above the maximum acceptable microbial limit were observed in 27/36, 11/36, and 20/36 AT samples, respectively, after T1 treatment.

During T2 water line sampling, no significant differences ($p \geq 0.05$) were observed in the median AMC, EB, and PS counts between the BT and AT samples (Table 1). No significant difference was observed between median AMC, EB, and PS count in poultry farms with private and public water supply. The highest median counts were for AMC counts in both BT (4.6 ± 1.55 $\log_{10}$ CFU/ml) and AT (4.7 ± 1.85 $\log_{10}$ CFU/ml) samples, followed by the PS counts in BT (3.5 ± 1.62 $\log_{10}$ CFU/ml) and AT (3.1 ± 2.05 $\log_{10}$ CFU/ml) samples The lowest counts were observed in the median EB counts of BT (2.4 ± 1.63 $\log_{10}$ CFU/ml) and AT (1.6 ± 0.42 $\log_{10}$ CFU/ml) samples. Higher median AMC, EB, and PS counts were detected in AT samples in poultry farms with public water supply.

After T2 water line treatment, high (>4.0 $\log_{10}$ CFU/ml) AMC, EB, and PS counts from BT samples decreased below the maximum acceptable microbial limit in 8/33, 5/33, and 14/33 AT samples, respectively (Supplementary Table S3). The AMC, EB, and PS counts below the microbial limit were detected in 4/33, 25/33, and 10/33 samples in both BT and AT, respectively. The AMC, EB, and PS counts remained above the maximum acceptable microbial limit in 21/33, 3/33, and 9/33 AT samples, respectively, after T2 water line treatment.

The impact of T1 and T2 water line treatment on private and public water supply was evaluated by calculating the $\log_{10}$ ratio from CFU $\log_{10}$ counts detected in BT and AT water samples (Table 1). No significant differences ($p \geq 0.05$) in $\log_{10}$ ratios were observed for AMC, EB, and PS counts after T1 and T2 water line treatment. The $\log_{10}$ ratio was not significantly different ($p \geq 0.05$) between private and public supplied poultry farms after T1 and T2 water line treatment. The median AMC, EB, and PS ratios after T1 waterline treatment were -0.2 ± 2.13, -0.6 ± 1.79, and 0.0 ± 2.26, respectively. The analysis of $\log_{10}$ ratios after T2 waterline treatment resulted in median values of -1.1 ± 2.13 for AMC, 0.0 ± 2.94 for EB, and 0.0 ± 3.12 for PS counts. Although $\log_{10}$

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TABLE 1 The median aerobic mesophilic count (AMC), *Enterobacteriaceae* (EB), and *Pseudomonadaceae* (PS) in poultry drinking water samples were determined before (BT) and after waterline treatment (AT) during T1 and T2 waterline treatment using culture-dependent methods.

Treatment (T)	Water supply	Median AMC log_{10} CFU/ml		Median AMC log_{10} ratio	Median EB log_{10} CFU/ml		Median EB log_{10} ratio	Median PS log_{10} CFU/ml		Median PS log_{10} ratio	*Campylobacter* spp.	
		BT	AT		BT	AT		BT	AT		BT	AT
1	Private	5.8 ± 1.30	5.4 ± 1.99	−0.5 ± 2.37	3.6 ± 2.13	1.6 ± 0.53	−1.1 ± 1.85	4.9 ± 1.30	3.7 ± 2.01	−0.7 ± 2.71	0/15	0/15
1	Public	5.9 ± 0.81	6.4 ± 0.88	−0.2 ± 1.68	3.5 ± 2.13	3.2 ± 2.77	−0.6 ± 1.01	4.6 ± 1.63	5.3 ± 1.95	0.3 ± 2.14	1/21	0/21
2	Private	5.0 ± 1.47	4.1 ± 0.82	−1.1 ± 1.68	1.3 ± 0.00	1.3 ± 0.00	0.0 ± 0.00	3.1 ± 2.11	2.5 ± 1.84	−1.8 ± 2.00	0/9	0/24
2	Public	4.5 ± 1.54	4.8 ± 2.15	−1.2 ± 2.88	2.6 ± 1.36	1.9 ± 0.83	−0.3 ± 3.74	3.7 ± 1.47	3.4 ± 1.54	0.8 ± 3.83	0/9	0/24
Total after T1		5.9 ± 1.02	6.0 ± 1.17	−0.2 ± 2.13	3.6 ± 2.13	2.3 ± 1.52	−0.6 ± 1.79	4.7 ± 1.44	4.7 ± 1.44	0.0 ± 2.26	1/36	0/36
Total after T2		4.6 ± 1.55	4.7 ± 1.85	−1.1 ± 2.13	2.4 ± 1.63	1.6 ± 0.42	0.0 ± 2.94	3.5 ± 1.62	3.1 ± 2.05	0.0 ± 3.12	0/33	0/33

The AMC, EB, and PS values are provided as median values (log_{10} CFU/g) and standard deviations. The presence (+) or absence (−) of *Campylobacter* spp. in water samples identified by the multiplex PCR assay. MAD, median absolute deviation.

ratios between poultry farms with private and public water supplies were not significantly different, we observed higher median log_{10} reduction of AMC, EB, and PS counts at poultry farms with private water supply. During T2 water line treatment higher median log_{10} reduction was observed for AMC and EB counts at poultry farms with public water supply, while higher median log_{10} reduction for PS counts was observed in poultry farms with private water supply.

Out of the 14 poultry farms assessed, five farms exhibited microbial counts below the acceptable microbial limit (<4.0 log_{10} CFU/ml) subsequent to the T1 water line treatment (Figures 2A–C). Among these farms, three had a private water supply, while the remaining two had public water supplies. Notably, poultry farm 7, which had a public water supply, exhibited an AMC count below the maximum acceptable microbial limit in both BT and corresponding AT water sample. Furthermore, 11 poultry farms exhibited EB counts below the maximum acceptable microbial limit. Of these, nine poultry farms demonstrated EB counts below the microbial limit in both BT and corresponding AT samples. Additionally, among 14 poultry farms examined, a total of eight poultry farms exhibited PS counts below the microbial limit. Out of these, four poultry farms demonstrated PS counts below the microbial limit in both BT and corresponding AT samples. Among the poultry farms that underwent two samplings, poultry farms 12 and 13 exhibited AMC and PS counts exceeding the microbial limit in one of the sampling events. Furthermore, poultry farm 12 demonstrated EB counts above the microbial limit on one of two sampling occasions.

During T2 waterline treatment AMC counts below the microbial limit were observ ed in six out of 14 poultry farms (Figures 3A–C). Of these, two poultry farms demonstrated AMC count below the microbial limit in both BT and corresponding AT samples (Figure 3B). EB counts below the microbial limit were observed in 12 out of 14 poultry farms, and among them, nine poultry farms had EB counts below the microbial limit in both the BT and corresponding AT samples. Similarly, PS counts below the microbial limit were observed in ten from 14 poultry farms, and among them, three poultry farms demonstrated PS counts below the microbial limit in BT and corresponding AT samples. Among the poultry farms subjected to two samplings, poultry farm 18 demonstrated AMC, PS and EB counts below the microbial limit during one of the sampling occasions. However, after second sampling, the AMC load in water samples exceeded the microbial limit. Notably, the PS and EB counts remained below the microbial limit during both sampling occasions.

3.2 Bacterial isolate identification in poultry drinking water

Isolate taxonomic assignment was performed using partial sequencing of 16S rRNA gene. In the present study, isolate sequences showed ≥97.0% similarity to the reference sequence in the NCBI database. In BT samples, 123 isolates corresponded to 24 genera and 55 species, while in AT samples, the 113 isolates corresponded to 22 genera and 40 species. Further analysis of bacterial isolates revealed that in BT and AT samples, 43.1% ($n = 41$ isolates) and 36.3% ($n = 53$ isolates) of sequenced isolates were assigned to OP, found in 29/36 BT and 17/36 AT samples (Table 2). The isolates from BT samples contained OP represented by 16 genera and 19 species, while isolates from AT samples contained OP represented by 12 genera and 12 species OP. Furthermore, *C. jejuni* was detected using multiplex PCR in a single BT water sample from a poultry farm with a public water supply.

During the T2 water line treatment, 139 isolates in the BT corresponded to 26 genera and 46 species, whereas 96 isolates in AT samples corresponded to 21 genera and 33 species (Table 2). Among the sequenced isolates, 30.9% ($n = 43$ isolates) and 33.3% ($n = 33$ isolates) corresponded to OP, isolated from 20/33 BT and 14/33 AT samples, respectively. The OP in the BT samples comprised 10 genera, and 14 species, while the OP in the AT samples comprised 11 genera and 14 species. No *Campylobacter* spp. were detected in poultry drinking water samples during the T2 water line treatment.

Figures 4A,B represents the taxonomic classification of assigned isolate sequences at phylum, and genus level. The predominant phyla in BT and AT samples were *Pseudomonadota,* followed by *Bacillota, Actinomycetota*, and *Bacteroidota* (Figure 4A). The frequently isolated genera during both T1 and T2 water line treatment in BT and AT samples were *Aeromonas, Bacillus, Citrobacter, Enterobacter, Pseudomonas* and *Stenotrophomonas* (Figure 4B). Among these, *Pseudomonas* (BT 38.2%; AT 32.7%) and *Bacillus* (BT, 13.0%, AT, 11.5%) were most commonly observed genera during T1 water line treatment. Similarly, during T2 water line treatment, *Pseudomonas* (BT, 31.7%; AT, 33.3%) and *Bacillus* (BT, 10.1%; AT, 11.5%) were predominant genera in BT and AT samples. The Figure 4B depicts the percentage identification of other observed genera during T1 and T2 water line treatments. The majority of sequenced isolates classified as OP in BT and AT samples during T1 and T2 water belonged to the *Pseudomonas* spp., followed by *Stenotrophomonas* spp., *Citrobacter* spp., *Ochrobactrum* spp., and *Acinetobacter* spp. (Figure 4C).

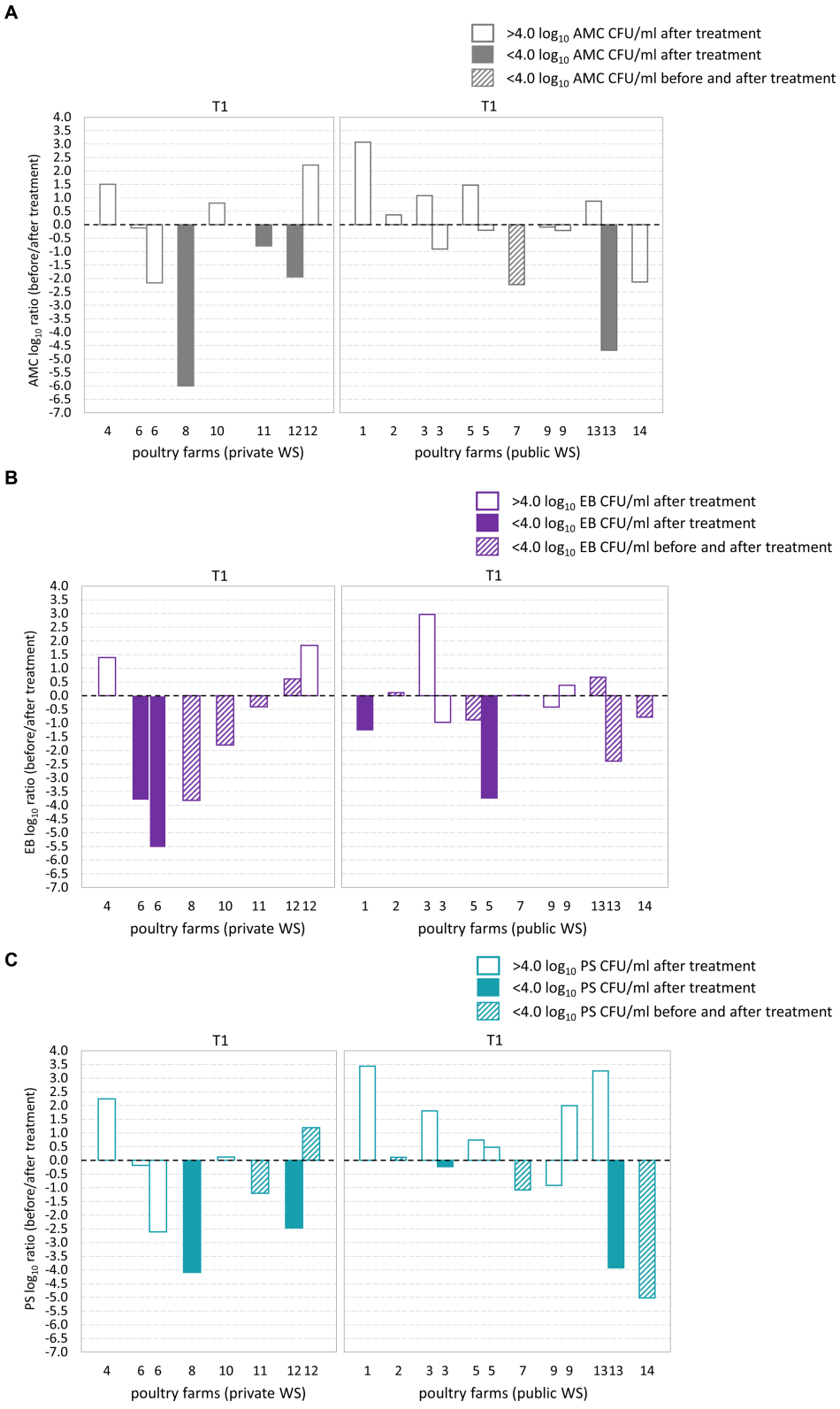

FIGURE 2
Log_{10} transformed average fold changes (before/after waterline treatment) obtained from aerobic mesophilic counts (AMC) **(A)**, *Enterobacteriaceae* (EB) **(B)**, and *Pseudomonadaceae* (PS) **(C)** in poultry drinking water. The x-axis indicates the comparison between poultry farms with private and public water supply (WS) after T1waterline treatment. The y-axis shows the log_{10} AMC, EB, and PS count ratio. The log_{10} AMC, EB, and PS ratio was not significantly different between poultry farms with private and public water supply. No significant differences were observed in the AMC, EB, and PS log_{10} ratio after T1 waterline treatment between poultry farms with private and public WS.

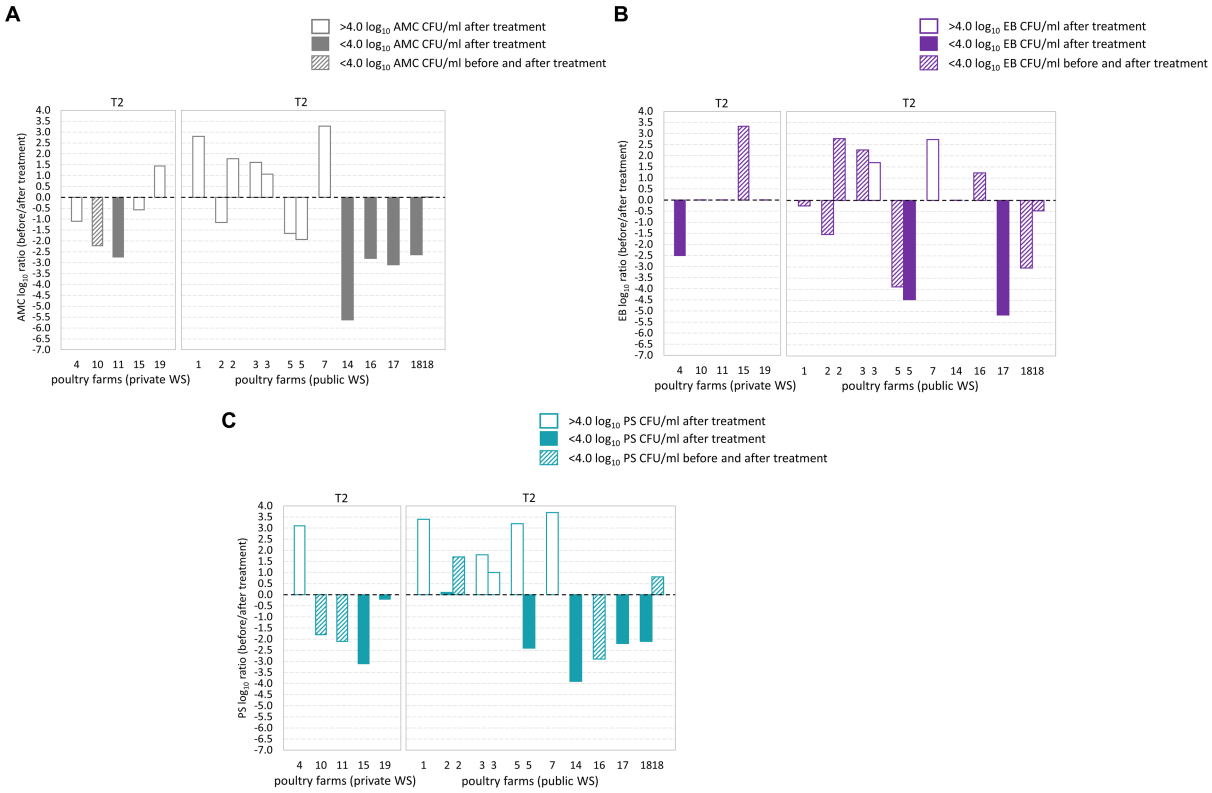

FIGURE 3
Log_{10} transformed average fold changes (before/after waterline treatment) obtained from aerobic mesophilic counts (AMC) **(A)**, *Enterobacteriaceae* (EB) **(B)**, and *Pseudomonadaceae* (PS) **(C)** in poultry drinking water. The x-axis indicates the comparison between poultry farms with private and public water supply (WS) after T2 waterline treatment. The y-axis shows the log_{10} AMC, EB, and PS count ratio. The log_{10} AMC, EB, and PS ratio was not significantly different between poultry farms with private and public water supply. No significant differences were observed in the AMC, EB, and PS log_{10} ratio after T2 waterline treatment between poultry farms with private and public WS.

TABLE 2 An overview of the number of isolate sequences assigned to the different phyla and genera using similarity cut-off of ≥97.0% after partial sequencing of 16S rRNA gene.

	T1		T2	
	Isolate diversity		Isolate diversity	
Sampling timepoint and isolate number	BT (*n* = 123)	AT (*n* = 113)	BT (*n* = 139)	AT (*n* = 93)
	n	*n*	*n*	*n*
Phylum	4	3	4	3
Genus	24	18	26	21
Opportunistic pathogens (≥97.0% sequence similarity)	*n*=53	53	43	33

The assigned bacterial isolate sequences encompass the classification of opportunistic pathogens present in water samples collected before (BT) and after (AT) the T1 and T2 water line treatment.

Furthermore, isolates of *Enterobacter* spp. and *Klebsiella* spp. genera were isolated during T1 and T2 sampling. The *Pseudomonas* spp. isolates identified as OP were most frequently observed bacteria sequences during both T1 (BT, 22/123 isolates; AT, 10/113 isolates) and T2 (BT, 13/139 isolates; AT, 10/96 isolates) sampling.

Before and after the T1 water line treatment, *Pseudomonas* spp. was isolated from BT and AT samples in 12/14 and 9/14 poultry farms, respectively (Table 3). Isolate sequences of OPs were detected in BT samples of 11 out of 14 poultry farms and in AT samples of 9 out of 14 poultry farms. Among the frequently observed genera before and after T2 treatment, the genus *Pseudomonas* was isolated from the BT and AT samples in 12 out of 14 poultry farms and 9 out of 14 poultry farms, respectively (Table 4). The OP were observed in 10 out of 14 poultry farms in BT samples and in 9 out of 14 poultry farms in AT samples after T2 water line treatment.

3.3 Antibiotic susceptibility patterns of bacterial isolates obtained from poultry drinking water

The susceptibility of bacterial isolates recovered from BT ($n = 14$) and AT ($n = 16$) water samples during T1 and T2 water line treatments to 18 antibiotic agents commonly used in poultry production was evaluated using Avian AVIAN1F Vet AST susceptibility plates (Table 5). The goal was to investigate AMR in the most frequently isolated OP isolates, including isolates belonging to *Pseudomonas* spp., *Stenotrophomonas* spp., *Ochrobactrum* spp., as well as AMR in specific waterborne OP important to human health, such as *Aeromonas* spp., *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp.

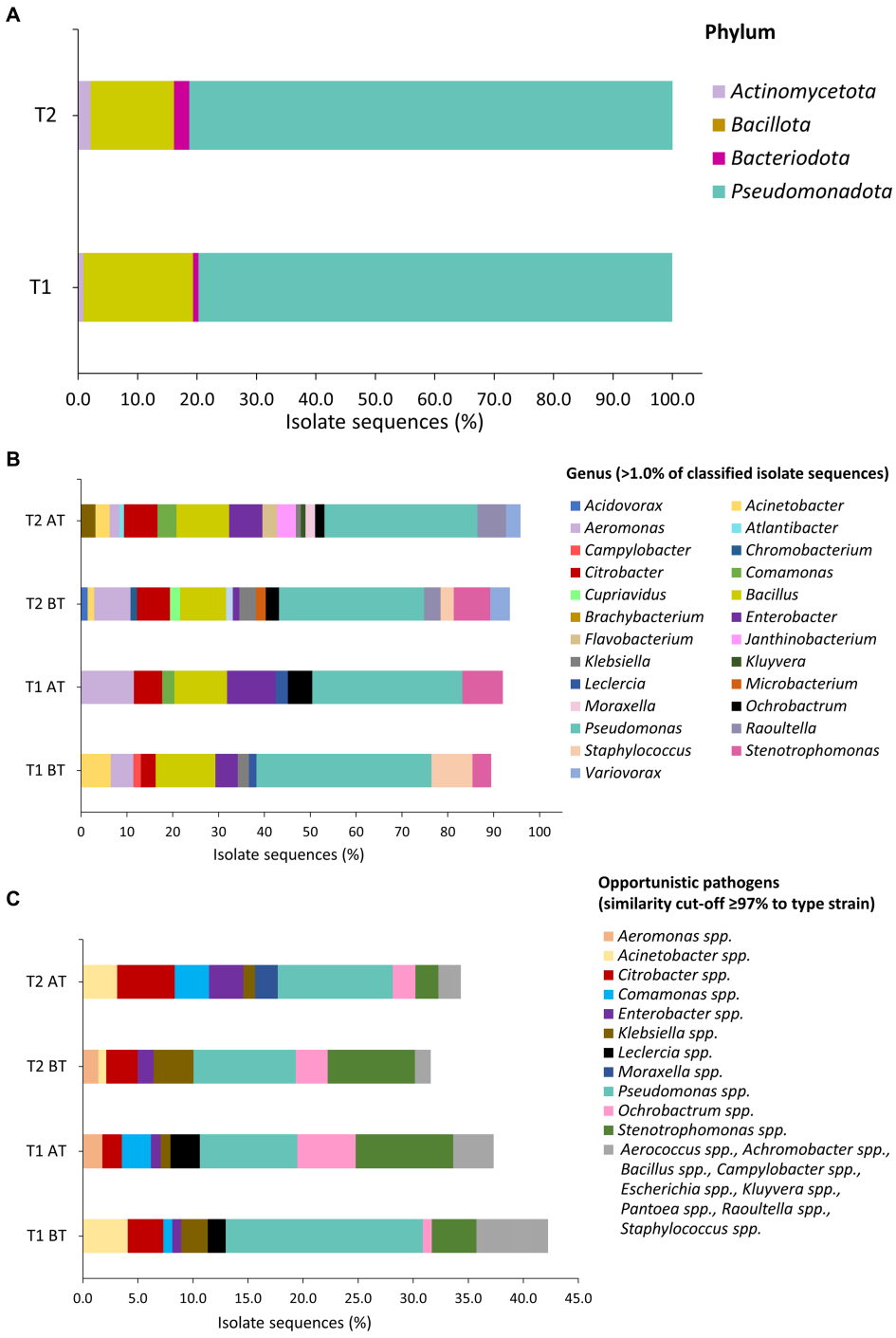

FIGURE 4
Taxonomic classification of isolates based on partial sequencing of 16S rRNA gene on phylum **(A)**, genus **(B)**, and opportunistic pathogens **(C)** level in water samples during T1 and T2 waterline treatments. Sequence similarity cut-off of ≥97.0% was applied for assignment of isolate sequences (1,040 to 1,120 bp fragment) to type strain was applied. **(C)** The bacterial sequences that were isolated from water samples one to two times are indicated by the grey color.

The highest level of AMR was observed against spectinomycin and sulfadimethoxin (90.0%; 27/30 isolates each), followed by ceftiofur (83.3%; 25/30 isolates), florfenicol (66.6%; 20/30 isolates), and neomycin (53.5%, 16/30 isolates). Further, some isolates were resistant to enrofloxacin (23.3%; 13/30 isolates), trimethoprim-sulfamethoxazole (23.1%; 3/13 isolates), sulfathiazole (20.0%; 6/30 isolates), streptomycin (16.7%, 5/30 isolates), gentamicin (13.3%; 4/30 isolates), and trimethoprim-sulfamethoxazole (10.0%, 3/30 isolates).

The MDR was exhibited among the isolates of *Pseudomonas* spp., (17/17 isolates), and *Stenotrophomonas* spp. (1/3 isolates), *Ochrobactrum* spp. (4/4 isolates), *Citrobacter* spp. (2/2 isolates), and *Enterobacter* spp. (1/2 isolates). All *Pseudomonas* spp. isolates showed

TABLE 3 The isolate diversity in poultry drinking water samples was assessed using partial sequencing of 16S rRNA gene of cultured isolates collected during chemical waterline treatment with 4.0 ppm active ClO_2 waterline treatment (T1) at poultry farms.

		Waterline treatment (T1)														Per sample isolation	
	Water supply	Private	Private	Private	Private	Private	Private	Public	Public	Public	Public	Public	Public	Public	Public	BT (n = 36)	AT (n = 36)
	Poultry farm	4	6	8	10	11	12	1	2	3	5	7	9	13	14		
	Water sample	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT		
Phylum (n = 4)	Genus (n = 29)																
Pseudomonadota (n = 20)	*Achromobacter*												1/1[1]			1	1
	Acinetobacter	1/0	1/0	2/0					4/0							5	0
	Aeromonas	0/1			0/1			1/0	2/9	1/0	0/2			2/0		5	5
	Atlantibacter													0/1		0	1
	Campylobacter													2/0		1	0
	Citrobacter									3/5	1/0		2/0			2	3
	Comamonas		0/2				1/0						0/1			1	2
	Enterobacter		2/0				1/1			0/1			2/10		1/0	4	6
	Escherichia		0/1													0	1
	Klebsiella									2/1	1/0					2	1
	Kluyvera				1/0											1	0
	Leclercia			1/0			1/3									2	1
	Ochrobactrum				0/1				0/5						1/0	1	3
	Pantonea				0/1											0	1
	Phytobacter												0/1		1/0	1	1
	Pigmentiphaga							1/0								1	0
	Pseudomonas	3/3	10/7		2/2	3/0	3/7	2/2	3/0	2/6	7/6	2/0	7/1	3/3		26	18
	Raoultella		1/0													1	0
	Rhizobium													0/1		0	1
	Stenotrophomonas			1/0			3/1		0/1		0/5		3/2	1/1	1/0	5	10
Actinomycetota (n = 2)	*Brachybacterium*													1/0		1	0
	Microbacterium			1/0												1	0
Bacillota (n = 5)	*Aerococcus*	1/0														1	0
	Bacillus			1/0	1/0	2/2			4/6	4/1	4/3				0/1	10	7
	Lysinibacillus						1/0									1	0
	Planococcus								1/0							1	0
	Staphylococcus		1/0		1/1		3/0		1/0					3/0	2/0	10	1
Bacteroidota (n = 2)	*Chryseobacterium*				0/1											0	1
	Sphingobacterium		1/0													1	0
Bacterial diversity on poultry farm		3/2	6/3	5/0	4/6	2/1	7/4	3/1	6/4	5/5	4/4	1/0	5/6	6/4	5/1		
Identified opportunistic pathogens		1/0	4/1	0/0	2/1	0/0	4/3	1/1	2/2	3/2	4/3	0/0	4/3	1/1	0/0	29/36	17/36

The occurrence of each genus in sample collected before treatment (BT, n = 36) and after treatment (AT, n = 36) was determined. The percentage of isolate occurrence was calculated based on cultured isolates from BT (n = 123) and AT (n = 113) samples. The isolate diversity at each poultry farm was evaluated in both BT and AT samples, and the presence of opportunistic pathogens was also determined. [1]Number of bacterial isolates isolated from BT and AT samples.

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TABLE 4 The isolate diversity in poultry drinking water samples was assessed using partial sequencing of 16S rRNA gene of cultured isolates collected during combined chemical (3.0% peroxyacetic acid [PAA] and 4.0 ppm active ClO_2) with mechanical (purging of waterlines with a high-pressure air pump) waterline treatment (T2) in poultry farms.

		Waterline treatment (T2)														Per sample isolation	
	Water supply	Private	Private	Private	Private	Private	Public	Public	Public	Public	Public	Public	Public	Public	Public	BS (n = 33)	AS (n = 33)
	Poultry farms	4	10	11	15	19	1	2	3	5	7	14	16	17	18		
	Water sample	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT		
Pylum (n = 4)	Genus (n = 33)																
Pseudomonadota (n = 24)	*Acidovorax*					0/2[1]						2/0			0/1	5	4
	Acinetobacter			1/0			0/1	1/0		0/2						2	2
	Aeromonas	0/1						4/1				1/0			6/0	7	2
	Atlantibacter						0/1									0	1
	Brevundomonas				0/1											0	1
	Buttiauxella							1/0								1	0
	Chromobacterium												2/0			2	0
	Citrobacter	0/2			0/3		1/0	0/1	3/0	4/0	1/1				1/0	8	4
	Comamonas							0/4								0	3
	Cupriavidus			1/0	1/0								1/0			3	0
	Enterobacter					1/0			1/3						0/4	2	3
	Janthinobacterium							0/1	0/3							0	3
	Klebsiella				0/1		3/0			2/0						2	1
	Kluyvera						0/1									0	1
	Moraxella								0/2							0	1
	Ochrobactrum							2/2							2/0	3	1
	Pantonea							0/2								0	1
	Phytobacter						1/0									1	0
	Pigmentiphaga														1/0	1	0
	Pseudaeromonas												1/0			1	0
	Pseudomonas	1/1	5/0	1/0	6/0	4/1	0/2	2/1	0/8	5/6	3/3	5/0	1/0	4/3	7/7	19	16
	Raoultella							1/0					4/0			3	0
	Stenotrophomonas			2/0	1/0	2/1			6/0	0/5						7	3
	Variovorax	2/0		2/0	1/0	0/1									1/2	5	2
Actinomycetota (n = 2)	*Brachybacterium*													2/0		1	0
	Microbacterium	1/0		1/0											1/0	3	0
Bacilliota (n = 4)	*Bacillus*	1/1		3/0		1/0		6/5	2/0	1/5						10	8
	Jeotgalicoccus														1/1	1	1
	Staphylococcus							1/0						2/0	1/1	3	1
	Trichococcus									1/0						1	0

(Continued)

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TABLE 4 (Continued)

		Waterline treatment (T2)														Per sample isolation	
	Water supply	Private	Private	Private	Private	Public	Public	Public	Public	Public	Public	Public	Public	Public	Public	BS ($n = 33$)	AS ($n = 33$)
	Poultry farms	4	10	11	15	19	1	2	3	5	7	14	16	17	18		
	Water sample	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT	BT/AT		
Pylum ($n = 4$)	Genus ($n = 33$)																
Bacteroidota ($n = 3$)	*Chryseobacterium*			1/0												1	0
	Flavobacterium						0/1		0/2			1/0				1	3
	Pedobacter									1/0						1	0
Bacterial diversity on poultry farm		4/4	1/0	9/0	4/3	4/4	3/5	8/8	4/5	6/4	2/2	4/0	5/0	3/1	9/6		
Identified opportunistic pathogens		0/1	0/0	2/0	1/1	2/0	1/1	2/2	3/2	3/2	1/1	1/0	0/0	1/1	2/3	19/33	14/33

The occurrence of each genus in sample collected before treatment (BT, $n = 33$) and after treatment (AT, $n = 33$) was determined. The percentage of isolate occurrence was calculated based on cultured isolates from BT ($n = 139$) and AT ($n = 96$) samples. The isolate diversity at each poultry farm was evaluated in both BT and AT samples, and the presence of opportunistic pathogens was also determined. [1]Number of bacterial isolates isolated from BT and AT samples.

resistance patterns exhibiting resistance to a minimum of four and a maximum of eight antibiotics. Tested *Stenotrophomonas* spp. isolates also demonstrated resistance patterns to a minimum of four and a maximum of six antibiotics. All tested *Ochrobactrum* spp. were resistant to four antibiotics. The isolates of *Citrobacter* spp. were resistant to six antimicrobial classes and nine different antibiotics. The isolates of *Enterobacter* spp. showed resistance patterns to a minimum of two and a maximum of four antibiotics. The isolates of *Klebsiella* spp. were resistant to five antibiotics, while *Aeromonas* spp. isolate was susceptible to all tested antibiotic agents.

4 Discussion

Providing poultry with water that meets the highest quality standards is essential to ensure the safety and quality of the products derived from these animals. The presence of high microbial loads and biofilms in the drinking water lines can have a negative effect on poultry health and performance (14). Moreover, when health issues arise within a poultry flock, antibiotics are often administered through drinking water. This practice increases the risk of antibiotic resistance within poultry farms, presenting a potential threat to both animal and human health (12).

We assessed microbial quality of poultry drinking water at the end of the drinking line based on established limits from previous studies, where AMC, EB, and PS counts below 4.0 $\log_{10}$ CFU/ml were deemed acceptable (4, 7, 12). At the end of the fattening period, AMC exceeded acceptable limits in most poultry farms tested, with similar trends observed for PS counts. However, EB remained within acceptable levels in the majority of farms. Environmental factors, such as ambient temperatures (±25°C), low water flow rates, pipeline installation type, and feed additives (often mixed with glucose) provided ample nutrients for bacteria, contributing to a high microbial load at the end of the fattening period (42). Poultry farms opt to chlorinate and/or acidify their drinking water systems due to the easy application, cost-effectiveness, and broad antimicrobial properties of these treatment systems (12). Additionally, mechanical cleaning helps remove biofilm from surfaces inside the drinking water system. Surprisingly, plate count analysis did not show a significant reduction of microbial load (AMC, EB, and PS counts) in AT samples after chemical water line treatment (T1) or combined chemical with mechanical treatment (T2). Unlike previous reports associating poultry farms with a private water supply with elevated microbial loads, we did not observe significant differences in microbial load between poultry farms with private or public water supplies (43). The microbial counts observed in our study were similar to those found on surfaces inside poultry house drinking water systems, which were typically above 6.0 $\log_{10}$ CFU (12). This suggests a limited disinfection effectiveness likely due to low concentration of applied disinfectant. Despite mechanical cleaning and subsequent disinfection, high microorganism levels persisted in the water lines, indicating that the disinfectant concentration post-mechanical treatment was insufficient to eliminate the majority of microorganisms. However, our study focused solely on microbiological parameters, overlooking vital factors such as water hardness, pH, temperature, and free ClO_2 residues within the water lines. This limited our ability to comprehensively evaluate the

TABLE 5 Antimicrobial resistance among bacterial isolates before (BT) and after (AT) waterline treatment to a panel of veterinary antimicrobials commonly used in the poultry production.

				Antimicrobial class[1] (in μg/ml):										
				Aminoglycosides				Fluoroquinolones	Cephalosporins	Tetracyclines	Phenicols	Sulfonamides		Diaminopyrimidine/ sulfonamides
Opportunistic pathogens[2]	Treatment[3]	Time-poin[4]	Isolates (*n*)	GEN	SPE	NEO	STR	ENR	XNL	TET and OXY	FFN	SDM	STZ	SXT
				≥8	≥64	≥32	≥1,024	≥2/1	≥4	≥8	≥8	≥256		≥2/38
Citrobacter spp.	1	BT	1	0/1	1/1	1/1	0/1	1/1	0/1	1/1	1/1	1/1	1/1	1/1
	2	AT	1	0/1	1/1	1/1	0/1	1/1	0/1	1/1	1/1	1/1	1/1	1/1
Enterobacter spp.	1	AT	1	0/1	0/1	0/1	0/1	0/1	1/1	1/1	1/1	1/1	0/1	0/1
	2	AT	1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	1/1	0/1	0/1
Klebsiella spp.	2	BT	1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1	1/1	1/1	0/1
Ochrobactrum spp.	1	AT	1	0/1	1/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1
	2	BT	2	0/2	2/2	2/2	0/2	0/2	2/2	0/2	0/2	2/2	0/2	0/2
	2	AT	1	0/1	1/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1
Pseudomonas spp.	1	BT	8	0/8	8/8	0/8	1/8	3/7	8/8	NA[5]	8/8	8/8	1/8	NA
	1	AT	6	0/6	6/6	6/6	2/6	2/6	2/6		6/6	6/6	1/6	
	2	BT	1	1/1	1/1	0/1	0/1	0/1	1/1		1/1	1/1	0/1	
	2	AT	2	0/2	2/2	0/2	1/2	0/2	2/2		2/2	2/2	0/2	
Aeromonas spp.	2	AT	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Stenotrophomonas spp.	1	AT	1	1/1	1/1	1/1	1/1	0/1	1/1	NA	0/1	0/1	0/1	0/1
	2	BT	1	0/1	1/1	1/1	0/1	0/1	1/1		0/1	1/1	1/1	1/1
	2	AT	1	1/1	1/1	1/1	0/1	0/1	1/1		0/1	0/1	0/1	0/1
BT (*n*/*N*)[6]			14	2/14	14/14	5/14	1/14	4/14	12/14	1/14	10/14	14/14	4/14	2/14
AT (*n*/*N*)			16	2/16	13/16	11/16	4/16	4/16	13/16	3/16	10/16	13/16	2/16	1/16
Total (*n*/*N*)			30	4/30	27/30	16/30	5/30	7/30	25/30	4/10	20/30	27/30	6/30	3/30

[1]The resistance breakpoints for selected antimicrobial classes represented by the antimicrobial agents in μg/ml for ≥8 gentamicin (GEN); ≥64 spectinomycin (SPE), ≥32 neomycin (NEO); ≥1,024 streptomycin (STR); ≥2/1 enrofloxacin (ENR); ≥4 ceftiofur (XNL); ≥8 tetracycline (TET) and oxytetracycline (OXY); ≥8 florfenicol (FFN); ≥256 sulfadimethoxine (SDM) and sulfathiazole (STZ); ≥2/38 trimethoprim-sulfamethoxazole (STX). [2]Bacteria species identified by partial sequencing of 16S rRNA gene. [3]Waterline treatment type. [4]Isolate identification in water sample before treatment (BT) and after treatment. [5]NA: not applicable, bacteria have intrinsic resistance against the antimicrobial agent. [6]*n*/*N*: number of isolates resistant to particular antimicrobial agent/total isolates tested.

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efficiency of the 4 ppm active ClO_2 and 3% PAA during water line treatments. Previous studies have highlighted the limited effectiveness of water line disinfection practices using oxidizing agents such as chlorine or hydrogen peroxide (12). This limitation primarily arises from applied concentrations being lower than recommended by suppliers, which is in alignment with our observations of high microbial load in AT samples. In addition, inconsistencies were noted in AT water samples among poultry farms that were sampled twice, emphasizing the need for frequent water quality checks in a closed system. Even with the addition of typical concentrations of hydrogen peroxide (25–50 ppm) and free chlorine (2–5 ppm) to poultry drinking water during fattening, biofilm formation was observed in minimally contaminated water (7). Therefore, regular monitoring of microbial water quality, combined with consistent water line treatment during the fattening period, is a crucial aspect of robust biosecurity programs at poultry farms. Moreover, specialized contractors have been noted to achieve more effective water line treatment compared to farmers (42, 44). Finally, Zou et al. (45) demonstrated a significant reduction of *E. coli*, *Salmonella*, *Staphylococcus aureus*, and mold in poultry drinking water after treatment with sodium dichloroisocyanurate, correlating positively with poultry health.

The presence of high microbial load in water samples led to a wide taxonomic variety among isolates in both BT and AT samples, ranging between 18 and 26 genera. While definitive taxonomic conclusions require further extensive studies, the frequent presence of genera such as *Aeromonas, Bacillus, Citrobacter, Enterobacter, Pseudomonas* and *Stenotrophomonas,* commonly associated with waste and surface waters, underscores an increased risk to both poultry and human health in this study (19, 46). Identification of genera, including *Pseudomonas, Stenotrophomonas*, and *Ochrobactrum,* were in line with the isolates found on surfaces in poultry drinking water system (12). The majority of the identified bacteria found at poultry farms independent of their water supply were OP, specifically those belonging to *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp. The OP belonging to *Pseudomonas* spp. are linked to secondary infections in both poultry and humans. In poultry, these infections can manifest as septicemia, skin lesion infections, and hemorrhagic pneumonia (47). In immunocompromised humans, they can lead to septicemia, pneumonia, and urinary tract infections (48). Previous studies have also emphasized an increased mortality rate in poultry following *P. aeruginosa* OP infection (49, 50). A previous study demonstrated enhanced adhesion to abiotic surfaces, tissue invasion through cytotoxic effects, resistance to 0.2 mg/mL chlorine, and increased AMR among *P. aeruginosa* isolates from water (51). Moreover, *Stenotrophomonas maltophilia* and *Ochrobactrum intermedium* are emerging human environmental pathogens causing infections, primarily in immunocompromised patients (52). *S. matophilia* and *P. aeruginosa* are often co-isolated from the lungs of cystic fibrosis patients, and previous research findings suggest that *S. maltophilia* modulates the virulence of *P. aeruginosa* in a multispecies biofilm (53). While *S. maltophilia* and *O. intermedium* have been recognized to cause infections in immunocompromised humans, no established link between water quality and disease development in poultry production involving these bacterial species has been reported yet. Nevertheless, notable characteristics of these bacteria, such as resistance to disinfection and heat, slow growth, and biofilm formation, emphasize the potential risk of poultry and farmer infection through direct contact with drinking water, along with the risk of cross-contamination of chicken meat products during post-slaughter processing.

During T1 water line treatment, *C. jejuni* was detected in one water sample collected before water line treatment at a poultry farm with a public water supply, while other analyzed samples tested negative. The detection of *Campylobacter* spp. in water depends on factors such as sample volume, sample number, and bacterial concentration (54, 55). Furthermore, *Campylobacter* spp. can enter a viable but nonculturable state (VBNC) under environmental stress, potentially hindering growth on conventional culture media due to limited metabolic activity (56). Consequently, *Campylobacter* spp. might have been overlooked in other analyzed water samples due to limitations in the processing method. These limitations include a small sample volume, the absence of water sample filtration, and the potential presence of *Campylobacter* spp. in the VBNC state, which cannot be detected using the ISObased methods used in the current study. While this approach may have led to missing *Campylobacter* spp., our assessment of bacterial load and diversity in the water samples examined provided a comprehensive insight into both quantitative and qualitative microbial content in poultry drinking water. Notably, previous research emphasizes that a significant presence of *Pseudomonas* spp. in poultry drinking water heightens the risk of *Campylobacter* spp. infection, as *Campylobacter* sp. isolates from poultry can persist for extended periods within *P. aeruginosa* biofilms in drinking water (57–59).

Previous studies have established poultry farms as significant reservoirs of antimicrobial resistance genes, contributing to the emergence of AMR and transmission dynamics of MDR bacteria at the humananimalenvironment interface (60–62). Our findings align with these observations, revealing MDR patterns in all tested isolates of both *Pseudomonas* spp. and *Ochrobactrum* spp. isolates from BT and AT water samples. Furthermore, a single *Stenotrophomonas* spp. from BT water sample exhibited MDR pattern. The consistent AMR patterns observed in both BT and AT water samples align with our observations of ineffective water line treatment characterized by limited disinfectant concentrations that allow for the survival and persistence of AMR bacteria within the water lines. The antimicrobials permitted for poultry treatment in Austria at the time of this study include enrofloxacin, doxycycline, trimethoprimsulfamethoxazole, amoxicillinclavulanic acid, colistin sulfate, tetracycline, and gentamicin (63–67). For the isolates we utilized in the AST, information or protocols regarding the current or past treatment of poultry on these farms were not available to the authors; therefore a detailed analysis of the potential causes of AMR in these isolates was not possible. The isolates from both BT and AT water samples exhibited increased resistance patterns to spectinomycin, sulfadimethoxin, ceftiofur, florfenicol, and neomycin, likely attributed to their widespread use in poultry health management on farms. This raises concerns, as elevated streptomycin resistance in *E. coli* isolates from broilers in several countries in Europe, including Poland, Germany, Great Britain, France and Spain was previously reported (68). Additionally, resistance to streptomycin and sulfadimethoxin was previously reported in *Salmonella* spp. isolates from poultry farms in Canada and the United States (69–72). Furthermore, these isolates exhibited resistance to ceftiofur and enrofloxacin, both of which are

recognized as top priority critically important antimicrobials by the World Health Organization (73). This antimicrobial resistance raises concerns, as it can be indirectly transmitted through horizontal gene transfer to *E. coli, Salmonella* spp., *Campylobacter* spp. and other potential poultry and human pathogens. Heinemann et al. (42) reported isolation of extendedspectrum betalactamaseproducing bacteria (ESBL) such as *P. aeruginosa*, *Enterobacter* spp., *Klebsiella* spp., and *Acinetobacter baumanni* from poultry drinking water lines and sprinkler systems. ESBL bacteria can hydrolyze extendedspectrum cephalosporins, monobactams, and penicillins and thus lead to elevated morbidity and mortality, further complicating therapeutic choices, particularly among elderly and immunocompromised individuals (74–76). The observed AMR resistance patterns in poultry drinking water isolates highlight the potential for acquiring antimicrobial resistance through wateradministered medication, posing a risk and limiting treatment options in both veterinary and human medicine (1, 42, 77–79).

The study emphasizes the persistent challenge of maintaining microbial quality in poultry drinking water. The high microbial load observed is attributed to established microbiota in the water system, resistant to suboptimal disinfectant concentrations used during cleaning. Furthermore, our findings suggest that current poultry treatment and antibiotic usage may elevate the presence of AMR bacteria in drinking water due to inefficient management. Addressing this issue necessitates regular water monitoring, consistent water line treatment, and improved farmer education. Enhancing understanding of biological processes in drinking water systems and microorganism viability can lead to better guidance on herd health and farm productivity. Identifying and mitigating onfarm water quality risks, including assessing waterline technologies affecting microbiota in drinking water and water lines, is essential for controlling pathogen and antibiotic transmission in poultry production.

5 Conclusion

In conclusion, the majority of poultry farms in Austria exhibited high microbial loads in drinking water, largely attributed to inadequate water line management practices, including the use of suboptimal disinfectant concentrations and inconsistent treatment. Notably, there were no significant differences observed between chemical and combined chemical and mechanical water line treatments. The prevalent microbiota in poultry included *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp. Moreover, these isolates from both before and after water line treatment samples displayed increased resistance patterns to commonly used antimicrobials to treat bacterial infections in poultry. Our results underscore the need for future studies to consider appropriate water supply management on poultry farms in terms of the One Health approach, to protect public health, and to raise awareness among farmers and veterinarians.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

AM: Data curation, Writing – original draft, Investigation, Methodology, Software, Visualization. MM: Data curation, Methodology, Writing – review & editing. KW: Data curation, Methodology, Writing – review & editing. AS: Methodology, Writing – review & editing, Investigation. IK: Investigation, Methodology, Writing – review & editing. CF: Writing – review & editing. IL: Formal analysis, Methodology, Writing – review & editing. MW: Conceptualization, Writing – review & editing. BS: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1254442/full#supplementary-material

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3.2 Characterization of *Leuconostoc carnosum* and *Latilactobacillus sakei* during Cooked Pork Ham Processing

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Characterization of *Leuconostoc carnosum* and *Latilactobacillus sakei* during Cooked Pork Ham Processing

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Abstract: Cooked ham is a popular, ready-to-eat product made of pork meat that is susceptible to microbial growth throughout its shelf life. In this study, we aimed to monitor the microbial growth and composition of nine vacuum-packed cooked ham lots using plate counting until the microbial limit of 7.4 $\log_{10}$ AMC/LAB CFU/g was exceeded. Eight out of nine lots exceeded the microbial limit after 20 days of storage. Lactic acid bacteria strains, particularly *Leuconostoc carnosum* and *Latilactobacillus sakei*, prevailed in vacuum-packed cooked ham. *Leuconostoc carnosum* 2 (Leuc 2) and *Latilactobacillus sakei* 4 (Sakei 4) were isolated from raw meat and the post-cooking area of the food processing facility. Carbohydrate utilization patterns of *Leuc. carnosum* PFGE types isolated from raw meat and the food processing environment differed from those isolated from cooked ham. These findings demonstrate how raw meat and its processing environment impact the quality and shelf life of cooked ham.

Keywords: lactic acid bacteria (LAB); brine cultures; microbial limit; genotypic diversity; carbohydrate utilization; food spoilage; food waste

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1. Introduction

Cooked ham is a refined ready-to-eat (RTE) product and represents 26% of delicatessen products sold in Europe [1]. The quality of the final product depends on the quality of raw pork meat and processing techniques used, including injection of brine, tumbling, and cooking at a core temperature of 66–75 °C [2,3]. Thermal processing is a crucial step in cooked ham production and has an important impact on microbiota selection [1]. To maintain microbiological and sensory stability during shelf life, vacuum packaging and cold storage are frequently utilized to prevent the growth of potential spoilage microorganisms, eliminating the need for preservatives, antioxidants, and stabilizers [4–6]. However, the growth of psychrotrophic, strictly, and facultative anaerobic lactic acid bacteria (LAB) during the cold storage of cooked ham can cause sensory changes (off-flavors and odors, discoloration, and gas and slime formation) and eventually lead to spoilage, rendering the product unsafe for consumption [7,8].

Factors that influence the survival of spoilage organisms during cooked ham production include the microbial quality of the raw meat, the duration of processing, and hygiene in the food processing environment (FPE) [9]. Although the cooking step at a core temperature of ≥70 °C reduces the bacterial load close to the detection limit of the used microbiological methods, some thermoduric LAB species, enterococci, and other

microbes may survive this process [10]. Furthermore, there is a risk of product recontamination with spoilage-associated bacteria in the post-cooking area during cooling, slicing, and packaging [11,12]. To counteract this, cooked ham is typically vacuum packed and refrigerated to prevent the growth of Gram-negative spoilage bacteria such as *Pseudomonas* and *Enterobacteriaceae* spp. [5,13]. However, the storage conditions of cooked ham, such as packaging, product composition, hygienic conditions during processing, and storage temperature, can allow LAB to grow to up to 8.0 log colony-forming units (CFU)/g within a few weeks after packaging [8,14,15].

Currently, LAB growth in conventionally processed cooked ham can lead to unacceptable levels and limit its shelf life [16–18]. LAB refers to a group of heterogeneous microbial species, such as *Leuconostoc carnosum*, *Leuconostoc gelidum*, *Latilactobacillus sakei*, *Lactobacillus curvatus*, *Carnobacterium divergens*, and *Carnobacterium maltaromaticum*. Some of these species can cause spoilage of the product, while others can contribute to product stabilization as bioprotective cultures with reduced spoilage capacities [10].

Several studies have addressed the cross-contamination of cooked ham from raw meat and food production [10]. Dušková et al. [16] focused specifically on the meat origin of LAB by isolating *Leuconostoc (Leuc.) carnosum* and *Leuc. pseudomesenteroides* from pork carcasses and cooked ham slices. It was observed that once LAB species are introduced into the meat production facility, psychrotrophic LAB species thrive at low temperatures and contribute to cross-contamination of cooked ham batches. Previous studies have reported the isolation of *Leuc. mesenteroides*, *Leuc. carnosum*, *Leuc. gelidum*, *Latilactobacillus* (*Lb.*) *sakei* from FPE and demonstrated their presence in cooked ham as a result of cross-contamination events from raw meat in the pre- and post-cooking areas of the FPE [9,11,12,16,19,20]. Furthermore, a slicing machine has been identified as a critical point for the introduction of LAB into cooked ham [9]. Once present in cooked ham, LAB show tolerance to anaerobic and low-temperature conditions, as well as the influence of sugar-enriched additives used during cooked ham production, which exert pressure on LAB growth and proliferation. The purpose of this study was to evaluate the microbial quality of cooked ham during its storage. Within this context, the monitoring of LAB and aerobic mesophilic counts (AMC) was conducted throughout the shelf life of cooked ham until the maximum acceptable concentration of 7.4 $\log_{10}$ CFU/g during cold storage (4 °C) was exceeded. This concentration is considered acceptable according to the AgrarMarkt Austria Marketing GmbH (AMA) quality seal label criteria, which serve as a national guideline for food quality [21]. In addition, the use of brine cultures (*Staphylococcus carnosus*, *Kocuria salsicia*, and *Latilactobacillus sakei*) during the raw meat brining step was also investigated with the potential to extend the shelf life of cooked ham. Raw meat and environmental samples were collected at different stages and screened for the presence of LAB. The most frequently isolated LABs, *Leuconostoc (Leuc.) carnosum* and *Latilactobacillus (Lb.) sakei*, of different origins (i.e., raw meat, production environment, and cooked ham) were identified on strain level. Subsequently, the utilization of common carbohydrates and enzymatic activity were assessed for the frequently isolated pulsed field gel electrophoresis (PFGE) types. Our objective was to use this knowledge to guide production facilities in optimizing their process hygiene practices, which can help prevent food waste and economic losses.

2. Materials and Methods

2.1. Industrial Ham Production and Processing

Investigated cooked ham was produced in an Austrian meat processing facility using fricandeau meat from the pork leg. Raw meats (RM) were obtained from Austrian slaughterhouses, where they were deboned and transported to meat processing facilities under refrigerated conditions (2–4 °C). In the ham production facility, the standard procedure involved storing the raw meat in the cold room within raw meat delivery boxes (RMDB) for up to five days until processing. A detailed overview of the conditions during the cooked ham production process is presented in Supplementary Table S1. Briefly, the manufacturing process included a brining step using 7.0 kg of ice, 64.6 kg of drinking water, 10 kg of

0.5% nitrite curing salt (E250), 7.5 kg of sodium nitrite (E250), 2.0 kg of triphosphates (E451), 0.4 kg of sodium ascorbate (E301), and an 8.5 kg compound mixture including 40% maltodextrin, 12% sugar, 8% dextrose, and a 5.1% spice mixture. The injection rate was 12%. After brining, the meat was transferred to tumbling machines and massaged, followed by a rest period to allow the diffusion of the brine into the meat. Subsequently, the tumbled ham was wrapped in a cellulose casing, molded, and transferred to the cooking chamber. During the cooking step, the temperature was gradually increased until the ham reached a core temperature of 72 °C, which was maintained for 30–60 min. After cooking, the ham was placed on a trolley and rapidly cooled down to a core temperature of 2–4 °C. The cellulose casing was removed, then the cooked hams were placed in vacuum-shrink foil bags (Sealed Air Corporation of Cryovac Inc., Aurora, IL, USA) using a Cryovac VS90 automatic belt feed vacuum packing machine (Cryovac Inc., Aurora, IL, USA) and subsequently vacuum packed with a Cryovac Sealed Air, S.R.L. Type ST98 (Cryovac Inc., Aurora, IL, USA) vacuum packaging machine at 4 °C. The cooked ham was stored at 4 °C in a temperature-controlled cold room. The commercial shelf life of vacuum-packed cooked ham is defined by the Austrian meat processing facility, which sets a sell-by date of up to 40 days after the day of packaging.

2.2. Study Design and Sampling

The local cooked ham production factory in Austria provided raw meat, cooked ham, and swab samples from the food processing environment (FPE) under real processing conditions between February and September 2021. The samples were categorized into raw meat (RM), FPE, and cooked ham (CH), and a detailed sample overview is provided in Table 1. Analysis using culture-dependent methods was conducted on nine vacuum-packed cooked ham lots (1–9) during cold storage at 4 °C. The study was divided into three phases.

Table 1. Overview of raw meat (RM; *n* = 12), food processing environment (FPE; *n* = 34) and cooked ham (CH; *n* = 122) sampling. Abbreviations: B: brine; BC: brine cultures; CB: cutting board; CC: cellulose casing; CH: cooked ham; G: gloves during packaging of the end product; HAT: ham after tumbling; RM: raw meat; RMDB: raw meat delivery box; T: trolley; TAS: tumbler after sanitation.

Lot	RM	BC	FPE									CH								
			Pre-Cooking					Post-Cooking				Storage Days								
	n = 12		*n* = 14					*n* = 20												*n* = 122
			RMDB	B	BC	HAT	TAS	CC	T	CB	G	0	4	6	9	11	15	20	33	
1		*no*										2				2		2		6
2		*no*										2				2		2		6
3		*no*										2				2		2		6
4	2	*no*										2	2	2		2				8
5	2	*no*										2	2		2	2				8
6	2	*no*										2		2		2	2	2		10
7	2	*no*		1			1	1	1	1	1	2	2	2	2	2	2	2		14
8	2	*no*		1				1	1	1	1	2	2	2	2	2	2	2	2	16
		yes		1	1			1	1	1	1	2	2	2	2	2	2	2	2	16
9	2	*no*	1	1		2	1	1	1	1	1	2	2	2	2	2	2	2	2	16
		yes		1	1	2		1	1	1	1	2	2	2	2	2	2	2	2	16

In the first phase, the microbial status of cooked ham during cold storage was evaluated by examining only the end product of lots 1 to 3. Consequently, the investigation of lots 1 to 3 did not encompass the raw meat used in the production of cooked ham.

The second phase involved the microbial analysis of vacuum-packed cooked ham lots 4 to 7, including the raw meat used in their production. The aim was to capture the rapid growth phase of the cooked ham microbiota until it exceeded the microbial limit of 7.4 $\log_{10}$ AMC/LAB CFU/g.

Phase three focused on cooked ham lots 8 to 9, where the raw meat was treated with brine cultures (*Staphylococcus carnosus*, *Kocuria salsicia*, and *Latilactobacillus sakei*) (Wiberg, FRUTAROM Savory Solutions Austria GmbH, Salzburg, Austria) during the brining step.

Both the cooked ham batches with and without the brine cultures were microbiologically analyzed throughout the shelf life.

The microbiological investigation of the vacuum-packed cooked hams (CH) comprised a total of 90 samples (lots 1 to 9). The samples were analyzed between 0 and 33 days, except for lots 8 and 9. The aim was to determine when the microbial limit of 7.4 $\log_{10}$ AMC/LAB CFU/g was exceeded during cooked ham cold storage. Duplicate samples from lots 1 to 3 were assessed at three timepoints: During phase one of the study, the general microbial status of cooked ham was assessed; therefore, the cooked ham samples were investigated at three different timepoints, including days 0, 11, and 20 of cooked ham storage. In phase two of the study, lots 4 to 7 were analyzed at shorter time intervals to capture the fastest growth phase of cooked ham microbiota until the microbial limit was exceeded. Therefore, there was a slight variation in sampling days until the microbial limit was reached. Cooked ham lots 8 to 9 were incubated until storage day 33 to describe the changes in microbial growth dynamics after exceeding the microbial limit. During the second and third phases of the study, swab samples were taken from the FPE to assess the influence of the hygiene conditions in the pre- and post-cooking areas of the FPE on the microbiota of cooked hams. Swab samples were taken by a trained employee of the cooked ham production facility along the processing line during the production of lots 7 to 9. The surfaces (10×10 cm^2) in FPE were sampled using sterile polyurethane sponges (World Bioproducts, Woodinville, WA, USA) and subsequently placed into a sterile plastic bag.

In the pre-cooking area, the swab samples of brine (B, $n = 5$) (200 mL) were collected in sterile containers with screw caps during the production of lots 7 to 9. To gain more detailed insight into meat-derived cross-contamination in the pre-cooking area during the production of lot 9, samples were taken from raw meat delivery boxes (RMDB; $n = 1$) and ham after tumbling (HAT; $n = 4$). In addition, swab samples were taken from the tumbler after sanitation (TAS; $n = 2$) before the start of production of lots 7 and 9 in order to estimate the cleaning efficiency of the tumbler, which was routinely performed once a week. In the post-cooking area, swab samples were taken from the cellulose casing used for the molding and cooking of the ham (PC; $n = 5$), the trolley used to cool down the cooked ham (T; $n = 5$), the cutting board (CB; $n = 5$), and the personnel gloves (G; $n = 5$) during the packaging of the end product during the production of lots 7 to 9.

The raw meat and FPE samples were processed immediately upon arrival. The Austrian meat processing facility supplied vacuum-packed cooked ham upon completion of each production lot. Subsequently, the cooked hams were stored in a refrigerated room maintained at a temperature of 4 °C until the day of analysis, when they were opened.

2.3. Microbiological Analysis and pH Measure

The quantification of aerobic mesophilic count (AMC) and LAB counts in raw meat, food processing environments, and cooked ham was carried out according to ISO reference methods (ISO 4833-2:2013, ISO 15214:1998) [22,23]. The counts of *Enterobacteriaceae* (EB) and *Pseudomonadaceae* (PS) in raw meat were determined according to ISO 21528-2:2017 [24]. Raw meat, ham after tumbling, and cooked ham samples (25 g each) were diluted in duplicate in 225 mL of buffered peptone water (BPW) (Biokar Solabia Diagnostics, Pantin, France) and homogenized in a laboratory mixer (Stomacher® bag; Seward Ltd., Worthing, West Sussex, UK) for 180 s. Environmental sponge samples (World Bioproducts, Woodinville, WA, USA) were diluted in 50 mL of BPW and manually homogenized for 1 min. Brine (200 mL) was centrifuged at 8000 rpm for 30 min at 4 °C (Thermo Scientific, Sorvall Lynx 4000 centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA) and the pellet was diluted in 45 mL BPW. Brine cultures (0.1 g) were diluted in 10 mL of BPW and vortexed until dissolved. Subsequently, serial ten-fold dilutions were prepared up to dilution -10^{10} in BPW. The dilutions (100 µL) were plated on Trypto-Caseine Soy Agar supplemented with yeast extract (TSAY) plates (Biokar Solabia Diagnostics, Pantin, France) and All-Purpose Tween (APT) agar plates (Merck, Darmstadt, Germany). For the first dilution, 1 mL of the sample was plated on TSAY, APT, and VRBG. The AMC count was

determined on TSAY agar plates that were incubated aerobically at 30 °C for 48 h. The LAB counts were determined on APT agar plates that were incubated microaerobically (Thermo Scientific CampyGen™ 2.5 L Sachet, Oxoid Ltd., Hampshire, UK) at 30 °C for 48 h. The *Enterobacteriaceae* (EB) and *Pseudomonadaceae* (PS) counts were determined on Violet Red Bile Glucose (VRBG) agar plates (Merck KgaA, Darmstadt, Germany) after aerobic incubation at 30 °C for 48 h. The EB and PS colonies on VRGB agar were differentiated by their ability to ferment glucose, resulting in pink colonies with or without precipitation and pale colonies for PS. Presumptive EB and PS isolates were confirmed using an oxidase reaction (BioMerieux, Marcy I'Etoile, France) and subjected to 16S rRNA gene sequencing (as described in Section 2.4). The minimum and maximum limits for the determination of the AMC, LAB, EB, and PS in the samples ranged between 10 and 300 CFU. During the analysis of cooked ham lots 8 to 9, the pH value was determined by taking at least three measurements using a pH meter (Professional Portable pH Meter, Hanna Instruments Inc., Woonsocket, RI, USA).

2.4. Isolate Collection and Identification

Up to five representative colonies from each TSAY (n = 270), APT (n = 280) or VRBG (n = 58) agar were subcultured on the corresponding medium. The purified isolates (n = *156* from raw meat, n = 223 from FPE; n = 229 from cooked ham) were cryopreserved at −80 °C in Brain Heart Infusion Broth (Biokar Solabia Diagnostics) supplemented with 25% (v/v) glycerol (Merck KgaA). The isolate list is provided in Table S2. DNA extraction was performed according to a protocol published by Walsh et al. [25]. Briefly, bacterial material (10 µL) from the agar plate was resuspended in 100 µL of 0.1 M Tris-HCl pH 7 buffer (Sigma Aldrich, St. Louis, MO, USA) and centrifuged at 15,000× g for 5 s (Eppendorf Centrifuge 5425, Hamburg, Germany). Subsequently, 400 µL of Chelex 100-Resin (Bio-Rad, Hercules, CA, United States) was added to the bacterial suspension and heated at 100 °C for 10 min on the block heater (Thermo ScientificTM block heater, Thermo Fischer Scientific Inc., Waltham, MA, USA). The suspension was subsequently centrifuged at 15,000× g for 5 s (Eppendorf Centrifuge 5425, Hamburg, Germany), and the supernatant (100 µL) was transferred to maximum recovery tubes (Corning Incorporated Life Sciences, Reynosa, Mexico) and stored at −20 °C until analysis. Identification of bacterial isolates was carried out by partial sequencing of the 16S rRNA gene using universal primer pairs 616F (5′-AGAGTTTGATYMTGGCTC-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) (both Microsynth AG, Blagach, Switzerland) as previously described [26,27]. A single PCR reaction (45 µL) contained diethylpyrocarbonate (DEPC)-treated water (Sigma Aldrich, St. Louis, MO, USA), 1 × buffer, 2 mM $MgCl_2$, 200 nM forward and reverse primers, 20 mM dNTP mix, 2 U of Platinum Taq DNA polymerase (Platinum™ Taq DNA Polymerase, DNA-free, Thermo Fisher Scientific Inc., Waltham, MA, USA), and 5 µL genomic DNA. The DNA amplification was performed in a T100TM Thermal Cycler (Bio-Rad, Hercules, CA, USA). Thermocycling conditions were 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s, and final elongation at 72 °C for 7 min. When 16S rRNA gene PCR yielded negative results, the presumptive fungi colonies were microscopically examined and then submitted to internal transcribed spacer 2 (ITS2) region sequencing. Presumptive fungi isolates were confirmed by sequencing the internal transcribed spacer 2 (ITS2) using primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [28]. A single PCR reaction (25 µL) consisted of diethylpyrocarbonate (DEPC) treated water (Sigma Aldrich), 1 × buffer, 2 mM MgCl2, 200 nM forward and reverse primers, 20 mM dNTP mix, 0.63 U of Platinum Taq DNA polymerase (Platinum™ Taq DNA Polymerase, DNA-free, Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1 µL template genomic DNA. The DNA amplification was performed in a T100TM Thermal Cycler (Bio-Rad, Hercules, CA, USA) at 95 °C for 5 min, 30 cycles at 94 °C for 40 s, 56 °C for 40 s, 72 °C for 60 s, and final elongation at 72 °C for 7 min. PCR products were previously evaluated by 1.5% agarose gel electrophoresis containing 1 × Tris-Acetate-EDTA buffer (TAE) and 3.5 µL peqGREEN DNA gel stain (VWR International, Radnor, PA, USA), at

120 V for 30 min. For the fragment length comparison, the DNA standard Thermo Scientific™ GeneRuler™ 1 kbp (Thermo Fisher Scientific Inc., Waltham, MA, USA) was applied. Subsequently, the obtained bacterial and fungal PCR amplicons were sent for purification and Sanger sequencing (LGC Genomics GmbH, Berlin, Germany). The bacterial genomic DNA PCR amplicons were sequenced using the 1492R (5′-GGYTACCTTGTTACGACTT-3′) primer from LGC Genomics (LGC Genomics GmbH, Berlin, Germany). The fungi PCR fragments were sequenced using the ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primer. The quality evaluation of the nucleotide sequences was performed using Finch TV 1.4.0 (Geospiza Inc. Seattle, WA, USA; https://digitalworldbiology.com/FinchTV, accessed on 25 March 2021). For bacterial datasets, the Nucleotide BLAST (Basic Local Alignment Search Tool) algorithm from the National Centre for Biotechnology Information was used for taxonomy assignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 30 March 2021). For fungi datasets, UNITE was used for taxonomy assignment (https://unite.ut.ee/, accessed on 30 July 2021). Sequences were assigned to genus or species level according to best matches and highest similarities (similarity cut-off ≥98%). The partial 16S rRNA gene sequence data of the isolates were deposited in the GenBank database under accession numbers OP263127–OP263616, while the fungi sequence data were under accession numbers OQ940410–OQ940442 (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 9 May 2023).

2.5. Molecular Subtyping of Leuconostoc carnosum and Latilactobacillus sakei

The *Leuconostoc (Leuc.) carnosum* (n = 144) and *Latilatcobacillus (Lb.). sakei* (n = 88) isolates were identified on a strain level using a molecular subtyping method. The detailed list of *Leuc. carnosum* (raw meat, n = 2; FPE, n = 20; cooked ham, n = 122) and *Lb. sakei* (raw meat, n = 7; FPE, n = 26; cooked ham, n = 55) selected for molecular subtyping is provided in supplementary Table S2. The *Leuc. Carnosum* and *Lb. sakei* isolates intended for pulse field gel electrophoresis (PFGE) analysis were grown on APT agar at 30 °C for 48 h under microaerobic conditions (Thermo Scientific CampyGenTM 2.5L Sachet, Thermo Fisher Scientific Inc., Waltham, MA, USA). Subsequently, bacteria were subcultured by inoculating 5 mL of de Man, Rogosa and Sharp (MRS) broth (Oxoid Ltd., Hampshire, UK) and grown overnight at 30 °C under microaerobic conditions. The preparation of plugs and the DNA restriction digestion were carried out as previously described with modifications [19,29]. Briefly, for plug preparation, cells were harvested from 2 mL of MRS broth after they reached an OD_{600} 0.9–1.5 (Oxoid Ltd., Hampshire, UK) by centrifugation at 8000× g for 5 min. The resulting pellet was resuspended in 1 mL of ice-cold PIV buffer (0.01 M Tris-HCl pH 7, 1 M NaCl; Sigma-Aldrich Corp., St. Louis, MO, USA). For the cell lysis, 240 µL of the bacterial suspension was transferred into a separate tube and mixed with 60 µL of lysozyme (10 mg/mL; Sigma-Aldrich Corp, St. Louis, MO, USA). The suspension was incubated for 30 min at 56 °C. After cell lysis, 25 µL of proteinase K (20 mg/mL) (Roche Diagnostics GmbH, Mannheim, Germany) was added to each suspension. Subsequently, the bacterial suspension (325 µL) was mixed with 1.2% (w/v) SeaKem Gold agarose (Lonza Group, Basel, Switzerland), which had previously been prepared in PIV buffer, and dispensed into the molds and left to solidify at room temperature. The second cell lysis step was performed by transferring the solidified plugs to 5 mL of cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8, 1% lauroylsarcosine, 0.1 mg/mL proteinase K; Sigma-Aldrich Corp., St. Louis, MO, USA) that were incubated overnight in shaking water bath at 54 °C (120 rpm). After cell lysis, the plugs were washed twice in 10 mL ddH2O and three times in 10 mL Tris EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0; Sigma Aldrich Corp.) for 10 min at 54 °C each. The restriction of *Leuc. carnosum* genomic DNA was performed with *ApaI* (0.25 U/µL; Thermo Fischer Scientific Inc., Waltham, MA, USA) at 25 °C for 4.5 h. The genomic DNA of *Lb. sakei* was digested with *AscI* (0.25 U/µL; Thermo Fischer Scientific Inc.) at 37 °C for 4.5 h. The plugs were loaded onto a 1% SeaKem Gold Agarose gel, and PFGE was performed in a CHEF DR III system (Bio-Rad Laboratories Inc., Hercules, CA, USA) in 0.5 × Tris borate EDTA (TBE) running buffer (45 mM Tris,

45 mM borate, 1 mM EDTA; Sigma-Aldrich Corp., St. Louis, MO, USA) for 22.5 h at 6 V/cm with a linear ramping factor and pulse times from 4.0 to 40.0 s at 14 °C and an included angle of 120°. After gel electrophoresis, the gel was stained with ethidium bromide (Sigma-Aldrich Corp.) and digitally photographed with a Gel Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The TIFF images were normalized with the BioNumerics 6.6 software package (Applied Math NV, Sint-Martens-Latem, Belgium) using the universal *Salmonella* ser. Braenderup H9812 standard. Pattern clustering utilized the unweighted pair group method with arithmetic mean (UPGMA) and the dice correlation coefficient with a position tolerance of 1.5%. PFGE types were considered identical when the patterns were indistinguishable. The Simpson's index of diversity was calculated using the Comparing Partitions online tool (http://www.comparingpartitions.info/index.php?link=Home, accessed on 6 June 2022).

2.6. Carbohydrate Utilization and Enzymatic Activity of Isolates

Isolates from raw meat (*Leuc. carnosum*, $n = 2$; *Lb. sakei*, $n = 5$), FPE (*Leuc. carnosum*, $n = 4$; *Lb. sakei*, $n = 19$), and cooked ham (*Leuc. carnosum*, $n = 45$; *Lb. sakei*, $n = 24$) as shown in the isolate list (Table S2) with identified PFGE types were tested for the utilization of 49 carbohydrates using API 50 CHL (API System, BioMerieux, Marcy I'Etoile, France), according to the manufacturer's instructions. *Leuc. carnosum* and *Lb. sakei* isolates recovered at the beginning (<6 days), during (9 to 11 days), and at the end (>20 days) of cooked ham storage were analyzed using API 50 CHL. The LABs were grown as described in Section 2.4. The isolates were inoculated into API strips and incubated for 48 h at 30 °C. Subsequently, the acid production from the supplied carbohydrates was determined as described by the manufacturer. For the evaluation of carbohydrate utilization patterns, we categorized the PFGE types into different biochemical profiles (BP) in dependence on their specific carbohydrate fermentation patterns and calculated the percentage of carbohydrates utilized per PFGE type.

The enzymatic activity of the identified distinct *Leuc. carnosum* ($n = 14$ isolates) *and Lb. sakei* ($n = 12$ isolates) PFGE types was analyzed using API ZYM (Bio Merieux, Lyon, France) as described by the manufacturer. The isolates selected for the API ZYM test are shown in the isolate list in Supplementary Table S2. The LABs were grown as described in Section 2.4. Subsequently, the suspension was spotted (45 µL) into wells and incubated at 25 °C for 4 h. Then, one drop of each of the kit reagents, ZYM-A and ZYM-B, was added to each well. The wells were incubated for 5 min, allowing the reactions to develop. The enzymatic activity was determined as described by the manufacturer.

2.7. Statistics

Using the program IBM SPSS v28, a binary logistic regression analysis was applied to estimate the probability of reaching the limit of 7.4 $\log_{10}$ CFU/g (AMC and LAB) using storage day as the predictor. A p-value below 5% ($p < 0.05$) was seen as significant.

3. Results

3.1. Microbial Characterization of Raw Meat and Cooked Ham during Storage

To assess the level of microbial contamination in raw meat utilized for cooked ham production, the counts of AMC, *Enterobacteriaceae* (EB), and *Pseudomonadaceae* (PS) were evaluated in lots 4 to 9. The AMC load in raw meat ranged from 3.4 ± 0.05 to 5.9 ± 0.43 $\log_{10}$ AMC CFU/g, with the highest counts detected in lots 4, 7, and 9 (Table 2). The counts of LAB ranged from 2.6 ± 0.28 to 5.8 ± 0.07 $\log_{10}$ CFU/g, with the highest counts detected in lot 9. The EB counts in raw meat from lots 4, 5, 7, and 9 ranged from 1.1 ± 1.29 to 4.7 ± 0.13 $\log_{10}$ CFU/g, while the PS counts from lots 5, 6, 7, and 9 ranged from 1.7 ± 0.40 to 4.7 ± 0.33 $\log_{10}$ CFU/g. No EB growth was detected in raw meat lots 6 and 8, while no PS growth was detected in lots 4 and 8. Of particular interest was the isolation of *Leuc. carnosum* and *Lb. sakei* from raw meat, as these were two primary bacterial species identified during the cooked ham storage. *Leuc. carnosum* was isolated from raw

meat lots 5 and 7 (16.7%; n = 2/12; Table 2), while *Lb. sakei* was isolated in lots 4 and 8 (33.3%; n = 4/12; Table 2). Additionally, other frequently isolated genera from raw meat were spoilage bacteria belonging to the genera *Aeromonas*, *Brochothrix*, *Carnobacterium*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* (Table S3). Yeasts, including *Candida*, were isolated from raw meat in every lot except for lot 5, whereas *Yarrowia* was isolated only from lot 6 (Table S3).

Table 2. Average aerobic mesophilic count (AMC), lactic acid bacteria (LAB), *Enterobacteriaceae* (EB) and *Pseudomonadaceae* (PS) counts are provided as mean values ($\log_{10}$ CFU/g) and standard deviations in raw meat (RM) (n = 12) used for cooked ham production (lots 4–9). The presence, absence, or partial presence of specific microorganisms in the samples is indicated as follows: (+) presence in two biological replicates; (+/−) presence in one biological replicate; or (−) absence from two biological replicates *of Leuconostoc (Leuc.) carnosum*, *Latilactobacillus (Lb.) sakei* and other bacteria identified with partial 16S rRNA gene sequencing.

	Mean $\log_{10}$ CFU/g					
Lot	**AMC**	**LAB**	**EB**	**PS**	***Leuc. carnosum***	***Lb. sakei***
4	5.4 ± 0.75	3.2 ± 0.51	4.0 ± 0.62	<1.0	−	+
5	4.8 ± 0.17	3.4 ± 0.30	1.1 ± 1.29	2.3 ± 0.62	+/−	−
6	4.1 ± 0.27	2.6 ± 0.28	<1.0	1.7 ± 0.40	−	−
7	5.7 ± 0.08	4.0 ± 0.37	4.1 ± 0.44	4.6 ± 0.42	+/−	−
8	3.4 ± 0.05	3.0 ± 0.21	<1.0	<1.0	−	+
9	5.9 ± 0.43	5.8 ± 0.07	4.7 ± 0.13	4.7 ± 0.33	−	−
Percentage (%) (n/N)					16.7 (2/12)	33.3 (4/12)

A total of 90 samples from nine different cooked ham lots (1 to 9) were microbiologically evaluated from the date of packaging (day 0) until the point when the maximum acceptable microbial limit of 7.4 AMC/LAB $\log_{10}$ CFU/g was exceeded during the cold storage at 4 °C (Table 3). At the beginning of the storage (day 0), the microbial counts were below the detection limit (<1.0 $\log_{10}$ CFU/g) for most lots except for lot 1 (3.5 ± 0.01 AMC and LAB $\log_{10}$ CFU/g) and lot 4 (1.3 ± 0.43 AMC $\log_{10}$ CFU/g) (Table 3). During the storage period, eight of nine lots exceeded the microbial limit on day 20, with lot 6 being the only exception, which remained below the detection limit until day 20 of storage (1.7 ± 0.57 AMC $\log_{10}$ CFU/g). Specifically, lots 1, 4, and 5 exceeded the limit on day 11 of storage, while lots 7, 8, and 9 exceeded the limit on day 15 of storage. Lots 2 and 3 exceeded the limit on day 20 of storage. Microbial counts for AMC and LAB reached a plateau between days 15 and 33 of storage, ranging between 7.9 and 9.2 $\log_{10}$ CFU/g for AMC and 7.8 and 9.1 $\log_{10}$ CFU/g for LAB.

The probability of achieving 7.4 $\log_{10}$ CFU/g AMC reaches 50% on day 11 and 100% on day 15. However, logistic regression showed no significant OR. When the same is performed for LAB, the probability of achieving 7.4 $\log_{10}$ CFU is 19% on day 11, 58% on day 15, and 93% on day 20, with a significant OR of 1.57 (p = 0.002). During the storage of cooked ham lots 1 to 9, the predominant microbial flora consisted of 62.2% *Leuc. carnosum* (n = 56/90) and 37.8% *Lb. sakei* (n = 34/90). Other species of LAB (7.8%; n = 7/90), including *Latilactobacillus graminis*, *Leuconostoc mesenteroides*, and *Weissella viridescens*, were only isolated at the beginning of the storage (Table S3). In addition, other non-LAB bacteria (13.3%; n = 12/90) belonging to the genera *Pseudomonas*, *Kocuria*, *Corynebacterium*, *Bacillus*, and *Staphylococcus* were also isolated at the beginning of the storage (Table S3). Yeasts, including *Cutaneotrichosporon* and *Filobasidium*, were isolated at the beginning of storage lot 9 (Table S3).

Table 3. Average aerobic mesophilic count (AMC) and lactic acid bacteria (LAB) of cooked ham samples (lots 1–9, n = 90) are provided as mean values and standard deviations. The presence, absence, or partial presence of specific microorganisms in the samples is indicated as follows: (+) presence in two biological replicates; (+/−) presence in one biological replicate; or (−) absence from two biological replicates. *Leuconostoc (Leuc.) carnosum*, *Latilactobacillus (Lb.) sakei*, other LAB (e.g., *Latilactobacillus graminis*, *Leuconostoc mesenteroides*, *Weissella viridescens*), and non-LAB (e.g., genus *Bacillus*, *Corynebacterium*, *Enterococcus*, *Pseudomonas*, *or Staphylococcus*) were identified with partial 16S rRNA gene sequencing.

Lot	Storage Days	Mean Log_{10} CFU/g		*Leuc. carnosum*	*Lb. sakei*	Other LAB	Non-LAB Bacteria
		AMC	LAB				
1	0	3.5 ± 0.01	3.5 ± 0.00	+	+	−	+
	11	7.8 ± 0.02	7.8 ± 0.06	+	+	−	−
	20	8.3 ± 0.01	8.3 ± 0.01	+	+	−	−
2	0	<1.0	<1.0	−	−	−	+
	11	7.3 ± 0.32	7.3 ± 0.35	+	+	−	−
	20	7.9 ± 0.04	7.8 ± 0.03	+	+	+/−	−
3	0	<1.00	<1.0	−	−	−	+
	11	6.8 ± 0.02	6.8 ± 0.07	+	−	+/−	−
	20	8.3 ± 0.06	8.3 ± 0.08	+	+	−	−
4	0	1.3 ± 0.43	<1.0	−	−	−	+
	4	3.1 ± 0.47	2.9 ± 0.20	+	−	+	+/−
	6	3.8 ± 0.23	3.7 ± 0.21	+	+	−	−
	11	7.6 ± 0.16	7.4 ± 0.42	+	+	−	−
5	0	<1.0	<1.0	−	−	−	−
	4	<1.0	<1.0	−	−	−	−
	9	6.9 ± 0.02	7.0 ± 0.33	+	+	−	−
	11	8.0 ± 0.27	7.7 ± 0.03	+	+	−	−
6	0	<1.0	1.0 ± 0.03	−	−	−	−
	6	<1.0	<1.0	−	−	−	−
	11	<1.0	<1.0	−	−	−	−
	15	<1.0	<1.0	−	−	−	−
	20	1.7 ± 0.57	<1.0	−	−	−	−
7	0	<1.0	<1.0	−	−	−	−
	4	1.7 ± 0.20	1.9 ± 0.21	+	−	−	−
	6	4.3 ± 0.34	4.2 ± 0.21	+	+	−	−
	9	4.6 ± 0.20	5.0 ± 0.39	+	+	−	−
	11	7.4 ± 0.01	7.2 ± 0.26	+	+	−	−
	15	7.9 ± 0.59	8.3 ± 0.29	+	+	−	−
	20	8.2 ± 0.57	8.4 ± 0.04	+	+	+/−	−
8	0	<1.0	<1.0	−	−	−	−
	4	<1.0	<1.0	−	−	−	−
	6	2.1 ± 0.27	1.9 ± 0.19	+	−	−	−
	9	3.8 ± 0.14	3.7 ± 0.05	+	−	−	−
	11	4.6 ± 0.09	4.7 ± 0.09	+	−	−	−
	15	7.7 ± 0.05	7.7 ± 0.23	+	−	−	−
	20	9.2 ± 0.31	9.1 ± 0.29	+	−	−	−
	33	9.2 ± 0.64	9.1 ± 0.68	+	−	−	−
9	0	<1.0	<1.0	−	−	−	−
	4	1.5 ± 0.00	<1.0	−	−	−	+/−
	6	<1.0	<1.0	−	−	−	−
	9	1.4 ± 0.12	2.5 ± 1.50	−	−	−	+/−
	11	6.3 ± 0.82	5.6 ± 0.08	+	+	+/−	−
	15	7.5 ± 0.27	7.6 ± 0.40	+	+	−	−
	20	8.7 ± 0.08	8.7 ± 0.06	+	−	+/−	+/−
	33	7.6 ± 0.42	7.3 ± 0.70	+	−	−	−
Percentage (%) (n/N)				62.2 (56/90)	37.8 (34/90)	7.8 (7/90)	13.3 (12/90)

3.2. Effect of Brine Cultures on Cooked Ham Shelf Life

The microbiological evaluation of the potential of brine cultures to prolong shelf life was conducted during the production of lots 8 and 9 (n = 64 samples). In the brining step,

raw meat (n = 32) was treated with brine cultures, including *Staphylococcus carnosus*, *Kocuria salsicia*, and *Latilactobacillus sakei*, and compared to the batch (n = 32) without brine cultures. The results showed that in the case of lot 8, the batch with brine cultures exceeded the microbial limit on day 20 of storage, while the batch without brine cultures exceeded the limit on day 15 (Figure 1a). In addition, the pH value during the storage of cooked ham lots 8 and 9 was also monitored (Figure 1b). For lot 8 with brine cultures, the initial pH value was 6.61 ± 0.24 and decreased to a pH value of 5.88 ± 0.08 on day 33 of storage. For lot 8 without brine cultures, the initial pH value was 6.06 ± 0.08, and it decreased to a pH value of 5.62 ± 0.23 on day 33 of storage.

In lot 9, the batch with brine cultures exceeded the microbial limit on day 11 of storage, while the batch without brine cultures exceeded the limit on day 15 (Figure 1a). For lot 9 with brine cultures, the initial pH of 6.05 ± 0.04 increased to a pH value of 6.51 ± 0.02 during the cooked ham storage (day 11), and it decreased to a pH value of 5.98 ± 0.05 on day 33 of storage (Figure 1b). For lot 9 without brine cultures, the initial pH value of 5.91 ± 0.12 increased to a pH value of 6.14 ± 0.01 during cooked ham storage (day 11) and decreased to a pH value of 5.54 ± 0.31 on day 33 of storage.

During the storage of cooked ham lots with brine cultures, *Leuc. carnosum* was isolated from 50.0% (n = 16/32) of the samples, while *Lb. sakei* was isolated from a single sample on day 33 of storage in lot 8 (3.1%, n = 1/32) (Table S4). In lot 9 with brine cultures, other LAB species (9.4%, n = 3/32) and non-LAB bacteria (6.3%, n = 2/32) were sporadically identified during the storage, including *Carnobacterium* and *Leuconostoc* genera, as well as *Microbacterium*, *Pseudomonas*, and *Staphylococcus* genera (Table S3).

3.3. Microbial Load and LAB Occurrence in the Food Processing Environment

The microbial examination of samples from the food processing environment (FPE) was conducted to identify the presence of LAB in both the pre- and post-cooking areas (Table 4). The microbial loads on surfaces in the pre-cooking area ranged from 1.0 to 3.1 $\log_{10}$ CFU/cm^2 for AMC and LAB. The brine had microbial loads ranged from 1.2 to 3.8 $\log_{10}$ AMC CFU/mL and 2.7 to 3.9 $\log_{10}$ LAB CFU/mL. The brine cultures used during the brining step used for production of lots 8 and 9 had a concentration of 10.5 to 13.1 $\log_{10}$ AMC CFU/mL and 10.8 to 13.1 $\log_{10}$ LAB CFU/mL. The microbial counts in ham after tumbling ranged from 4.6 to 6.6 $\log_{10}$ AMC CFU/g and 3.9 to 6.5 $\log_{10}$ LAB CFU/g. In the post-cooking area, the microbial counts were low (1.0 to 2.4 $\log_{10}$ AMC CFU/cm^2 and 1.5 to 2.3 $\log_{10}$ LAB CFU/cm^2), with the highest counts identified on the cutting board.

Regarding the LAB in the FPE, both *Leuc. carnosum* and *Lb. sakei* (each 35.3%; n = 12/34) were isolated in the pre- and post-cooking areas, including the tumbler after sanitation, the ham after tumbling, the cellulose casing, the cutting board, and the gloves of the personnel (Table 4). In addition, *Leuc. carnosum* was found on the trolley in the post-cooking area, while *Lb. sakei* was identified as one of the species contained in the brine. During the cooked ham production, increased microbial diversity was observed on the cellulose casing, brine, and cutting board. Spoilage bacteria commonly associated with raw meat (e.g., *Brochothrix*, *Carnobacterium*, *Pseudomonas*, and *Staphylococcus* spp.) were isolated in the pre-cooking area, while mainly *Kocuria*, *Micrococcus*, *Pseudomonas*, and *Psychrobacter* spp. were isolated in the post-cooking area (Table S3). Yeast *Candida* was identified in the post-cooking area, including the cellulose casing, cutting board, and personnel gloves during the packaging of the end product (Table S3).

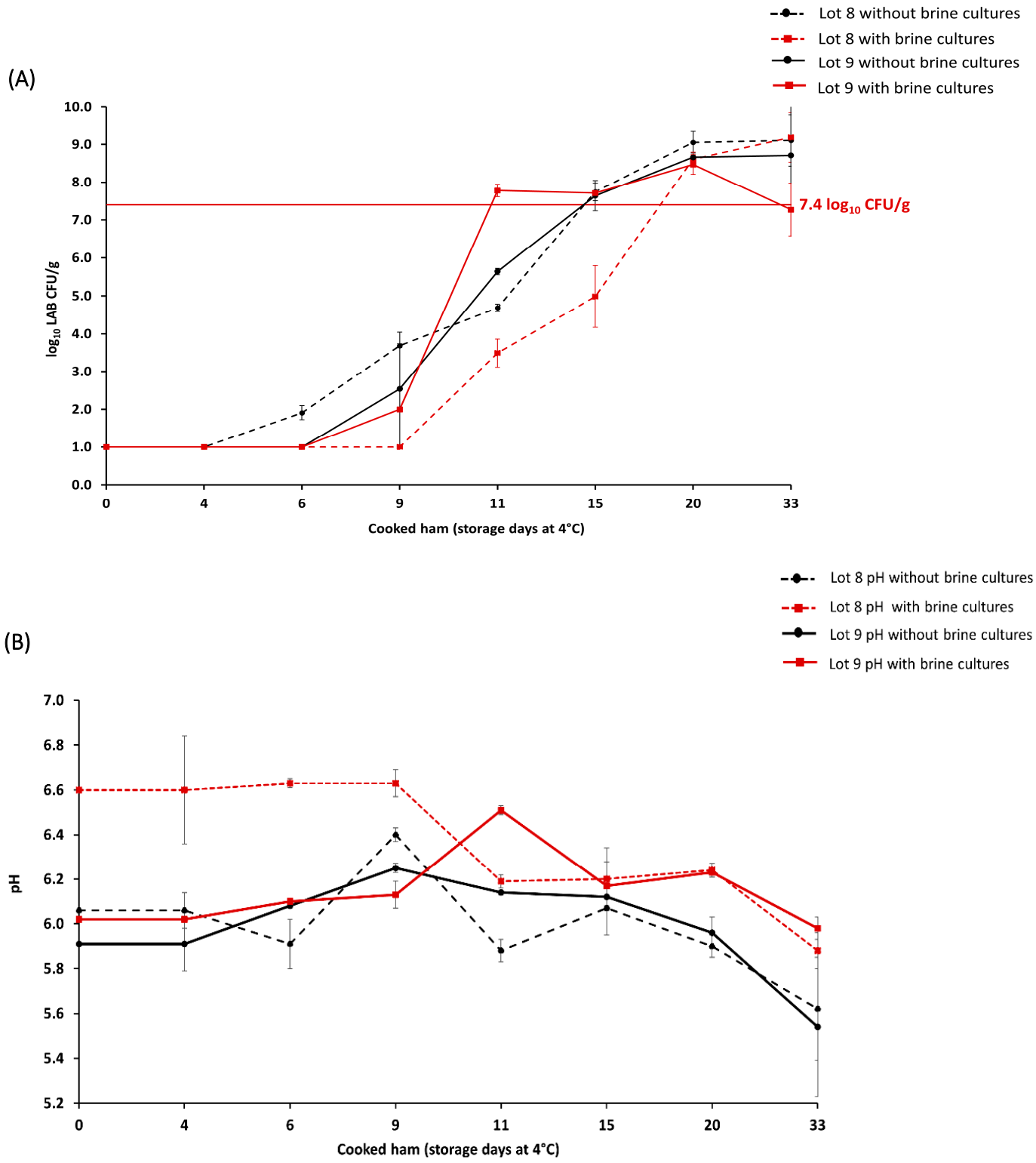


Figure 1. Average lactic acid bacteria (LAB) count (log_{10} CFU/g) in cooked ham lots 8 and 9 with (n = 32) and without (n = 32) brine cultures until day 33 of storage (**A**). The maximum acceptable microbial limit of ≥7.4 LAB log_{10} CFU/g during the cooked ham storage is marked with a horizontal, full red line. pH in cooked ham lots 8 and 9 measured until the day 33 of storage (**B**). The representation of cooked ham without brine cultures (●) is denoted in black, while samples with brine cultures (■) are shown in red.

Table 4. Average aerobic mesophilic count (AMC) and lactic acid bacteria (LAB) counts of the food processing environment (FPE) (lots 7–9; *n* = 34). The dashed line represents the distinction between pre- and post-cooking areas. The presence (+) or absence (-) of *Leuconostoc (Leuc.) carnosum*, *Latilactobacillus (Lb.) sakei*, and other bacteria (e.g., genus *Brochotrix*, *Carnobacterium Pseudomonas*, or *Psychrobacter*) were identified with partial 16S rRNA sequencing. Abbreviations: B: brine; BC: brine cultures; CB: cutting board; G: gloves during packaging of the end product; HAT: ham after tumbling; CC: cellulose casing; RM: raw meat; NA, not applicable; RMDB: raw meat delivery box; T: trolley; TAS: tumbler after sanitation.

FPE Sampling Area	Sample (log_{10} CFU/mL or cm^2)	AMC Log_{10} CFU/cm^2			LAB Log_{10} CFU/cm^2			*Leuc. carnosum*	*Lb. sakei*	Non-LAB Bacteria Number of Isolates
		Lot 7	Lot 8	Lot 9	Lot 7	Lot 8	Lot 9			
Pre-cooking	RMDB	NA	NA	3.1	NA	NA	3.1	−	−	4
	B	3.3	1.2–3.9	3.2–3.8	2.9	<1.0–3.7	2.7–3.9	−	+	14
	BC	NA	10.5	13.1	NA	10.8	13.1	−	+	2
	TAS	1.9	NA	3.0	1.9	NA	3.0	+	+	11
	HAT	NA	NA	4.6–6.6	NA	NA	3.9–6.5	+	+	10
Post-cooking	CC	1.7	1.2–1.5	1.3–1.4	<1.0	<1.0–1.1	<1.0–1.2	+	+	16
	T	<1.0	<1.0–1.2	<1.0–1.1	<1.0	<1.0	<1.0	+	−	11
	CB	1.4	1.7–2.4	1.9–2.4	1.5	<1.0–1.9	<1.7–2.3	+	+	13
	G	<1.0	1.1–1.7	1.0–1.4	<1.0	<1.0–1.2	<1.0	+	+	10
		Percentage (%) (n/N)						35.3 (12/34)	35.3 (12/34)	

3.4. Leuc. carnosum and Lb. sakei Strain-Level Characterization

Molecular strain-level analysis of the commonly isolated *Leuc. carnosum* and *Lb. sakei* LABs at different stages of cooked ham production (RM, FPE, and cooked ham) revealed that particular pulsed field gel electrophoresis types (PFGE types) were responsible for contaminating the final product. For the 144 *Leuc. carnosum* isolates (RM, n = 2; FPE, n = 20; cooked ham, n = 122), PFGE *ApaI* profiling resulted in 12 PFGE types and two subtypes, with a Simpson's diversity index of 0.668 (CI 95%, 0.494–0.782) (Figure 2a). In the pre-cooking area, diverse *Leuconostoc carnosum* (Leuc) PFGE types (Leuc 2, Leuc 6, Leuc 7, Leuc 8, Leuc 11, and Leuc 12) were present. In the post-cooking area, four different PFGE types (Leuc 1, Leuc 2, Leuc 3, and Leuc 8) were identified. The two most frequently isolated PFGE types in the post-cooking area, Leuc 2 and Leuc 3, were also the most abundant PFGE types in cooked ham. The PFGE type Leuc 2 (50%, n = 72/144) was identified in raw meat, the post-cooking area (cellulose casing and cutting board), and all cooked ham lots, while the PFGE type Leuc 3 (27.8%, n = 40/144) was isolated from all tested surfaces in the post-cooking area and six cooked ham lots (1, 3, 4, 7, 8, and 9). Additionally, five other PFGE types (Leuc 1, Leuc 4, Leuc 5, Leuc 9, and Leuc 10) and three subtypes (Leuc 1 ST, Leuc 5 ST, and Leuc 9 ST) were sporadically isolated during the cold storage of cooked ham lots (1, 2, 4, 8, and 9).

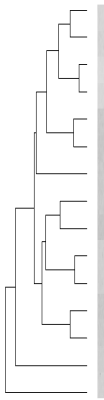
For the 88 *Lb. sakei* isolates (RM, n = 7; FPE, n = 17; brine cultures, n = 9; cooked ham, n = 55), subtyping resulted in 11 unique *AscI* profiles and one subtype, with a Simpson's diversity index of 0.839 (CI 95%, 0.809–0.870) (Figure 2b). In the pre-cooking area, eight *Latilactobacillus sakei* (Sakei) PFGE types (Sakei 2, Sakei 3, Sakei 4, Sakei 5, Sakei 7, Sakei 9, Sakei 10, and Sakei 11) and one subtype (Sakei 4 ST) were identified. In the post-cooking area, four different PFGE types (Sakei 3, Sakei 4, Sakei 8, and Sakei 10) were identified. The PFGE type Sakei 4 was the most frequently identified PFGE type (25.0%, n = 22/88), isolated from raw meat, the post-cooking area (cellulose casing and cutting board), and six cooked ham lots (2, 4, 5, 7, 8, and 9). The PFGE type Sakei 3 was the second most frequent type (14.8%; n = 13/88), isolated from raw meat, the cutting board, and five cooked ham lots (1, 2, 4, 5, and 8). The PFGE type Sakei 7 was identified as one of the isolates present in brine cultures. The PFGE type Sakei 7 (20.5%; n = 18/88) indicated dissemination along the cooked ham processing line (raw meat, brine, and ham after tumbling) during the production of lot 8 and 9 batches with brine cultures. Other PFGE types (Sakei 1, Sakei 5, Sakei 6, and Sakei 10) were sporadically identified during the storage of cooked ham lots (1 and 7).

3.5. Biochemical Characterization of Leuc. carnosum and Lb. sakei Isolates

In order to provide an in-depth characterization of LAB isolates, we conducted an analysis to determine whether the *Leuc. carnosum* and *Lb. sakei* isolates could ferment various types of sugar, such as mono-, di-, and trisaccharides, sugar acids, sugar alcohols, and glycosides, using the API 50 CHL test. Among the tested carbohydrates, all *Leuc. carnosum* isolates (n = 51) were found to utilize four specific carbohydrates, namely D-glucose, D-fructose, D-sucrose, and esculin (Figure 3a). Most of the isolates also utilized D-ribose, N-acetyl-glucosamine, D-trehalose, D-turanose, and gluconate, while a lower utilization ability was observed for methyl alpha D glucopyranoside, D-mannose, gentiobiose, cellobiose, and maltose. D-galactose, D-melibiose, D-melezitose, D-raffinose, glycogen, mannitol, salicin, amygdalin, and arbutin were found to be utilized specifically by certain isolates. Based on the carbohydrate utilization patterns of the tested *Leuc. carnosum* PFGE types were categorized into 13 different biochemical profiles (BP). Among these profiles, the PFGE types from the raw meat showed the most distinctive biochemical patterns compared to other *Leuc. carnosum* PFGE types. For example, the PFGE type Leuc 6 from raw meat showed the highest carbohydrate utilization of 37.7% (BP 13). The PFGE type Leuc 2 isolated from raw meat showed carbohydrate utilization of 20.4% (BP 5), which included fermentation of D-mannose, D-galactose, D-maltose, D-melezitose, and D-raffinose, which

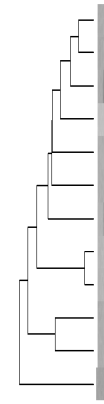
was not observed among the isolates from cooked ham. The carbohydrate utilization of PFGE type Leuc 2 isolates from cooked ham ranged between 14.3% (BP 1), 16.3% (BP 2), 18.4% (BP 4 and BP 6), and up to 20.4% (BP 7). The BP 1 and BP 2 profiles were shared by two PFGE types (Leuc 2 and Leuc 3), while the most frequently observed biochemical profile (BP 4) in cooked ham was shared by three PFGE types (Leuc 2, Leuc 3, and Leuc 4). The PFGE types (Leuc 7, Leuc 8, and Leuc 11) from the FPE showed three biochemical profiles (BP8, BP9, and BP10). The BP9 and BP10 were shared between different PFGE types (Leuc 8, Leuc 9, Leuc 9-ST, Leuc 1, Leuc 1-ST, Leuc 10, and Leuc 11) isolated from FPE and cooked ham. We observed no difference in carbohydrate utilization among isolates with identical PFGE types that were isolated at the beginning, during, and at the end of cooked ham storage.

(A)



PFGE *ApaI*	PFGE type	Isolates (n)								Lot	TOTAL Percentage % (n/N)	
		Pre-cooking area			Post-cooking area							
		RM	HAT	TAS	CC	T	CB	G	CH			
	Leuc 2	1			6		2		63	1*,2*, 3*, 4*, 5*, 7*, 8*, 9*	**50.0**	**(72/144)**
	Leuc 3				2	2	2	1	33	1*, 3*, 4*, 7*,8*, 9*	**27.8**	**(40/144)**
	Leuc 1				1				10	1*, 2*, 8, 9*	**7.6**	**(11/144)**
	Leuc 1 ST								1	9*	**0.7**	**(1/144)**
	Leuc 9								4	9*	**2.8**	**(4/144)**
	Leuc 9 ST								1	9*	**0.7**	**(1/144)**
	Leuc 8		1					1		9*	**0.7**	**(1/144)**
	Leuc 7			1						7	**0.7**	**(1/144)**
	Leuc 10								7	9*	**4.9**	**(7/144)**
	Leuc 5								1	8*	**0.7**	**(1/144)**
	Leuc 5 ST								1	8*	**0.7**	**(1/144)**
	Leuc 11			1						9	**0.7**	**(1/144)**
	Leuc 12			1						9	**0.7**	**(1/144)**
	Leuc 6	1								7	**0.7**	**(1/144)**
	Leuc 4								1	4*	**0.7**	**(1/144)**

(B)



PFGE *AscI*	PFGE type	Isolates (n)								Lot	TOTAL Percentage % (n/N)	
		Pre-cooking area					Post-cooking area					
		RM	B	BC	HAT	TAS	CC	CB	G	CH		
	Sakei 9		1							7	**1.1**	**(1/88)**
	Sakei 10			1				1		12	7*, 9	**15.9**	**(14/88)**
	Sakei 6									2	7*	**2.3**	**(2/88)**
	Sakei 2	1								2	**1.1**	**(1/88)**
	Sakei 7	4	1	9	4					8, 9	**20.5**	**(18/88)**
	Sakei 3	1						1		11	1*, 2*, 4*, 5*, 8*	**14.8**	**(13/88)**
	Sakei 5				1					1	1*	**1.1**	**(1/88)**
	Sakei 4	1					1	1	1	18	2*, 4, 5*, 7*, 8*, 9*	**25.0**	**(22/88)**
	Sakei 4 ST		1							7	7	**1.1**	**(1/144)**
	Sakei 8							2		1	7	**2.3**	**(2/88)**
	Sakei 1									11	1*, 3, 4, 5	**12.5**	**(11/88)**
	Sakei 11					2					9	**2.3**	**(2/88)**

Figure 2. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram with dice coefficient and 1.5% position tolerance among *XbaI* and *AscI* pulsed-field gel electrophoresis (PFGE) patterns of *Leuconostoc (Leuc.) carnosum* (**A**) and *Latilactobacillus (Lb.) sakei* (**B**) isolates from raw meat, food processing environments, and cooked ham. Abbreviations: B: brine; BC: brine cultures; CB: cutting board; CH: cooked ham G: gloves during packaging of the end product; HAT: ham after tumbling; CC: cellulose casing; RM: raw meat; RMDB: raw meat delivery box; T: trolley; TAS: tumbler after sanitation; *: represents the isolation of the PFGE type from cooked ham.

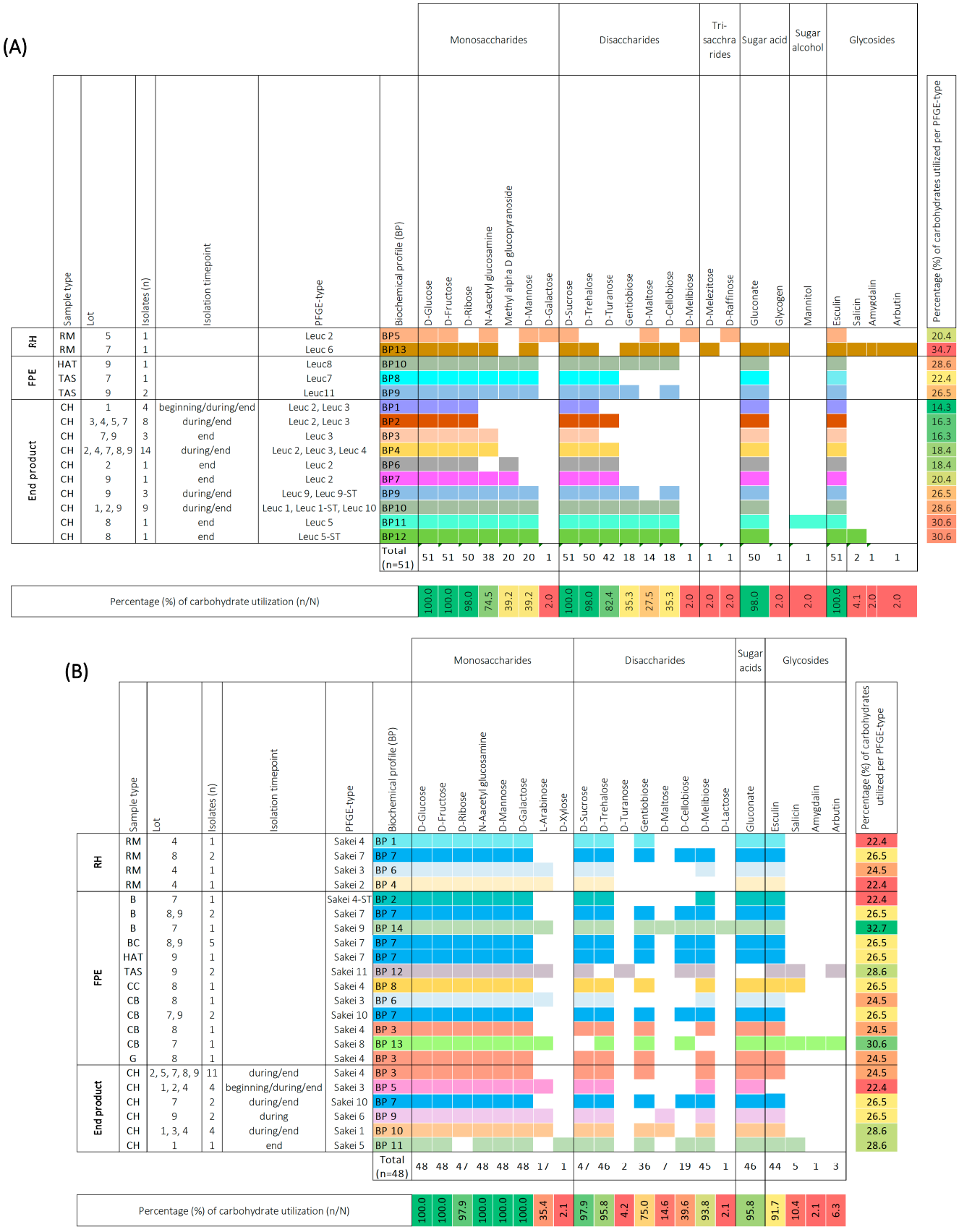


Figure 3. Biochemical profiles were generated based on the 49-carbohydrate utilization ability of *Leuconostoc (Leuc.) carnosum* (*n* = 51) (**A**) and *Latilactobacillus (Lb.) sakei* (n = 48) (**B**) isolates. All carbohydrate

substrates (API 50 CHL) were categorized into monosaccharides, disaccharides, trisaccharides, sugar acids, sugar alcohols, and glycosides. The isolate's ability to utilize a carbohydrate is represented as a positive (coloration) or negative (white color) reaction. The carbohydrates that were not utilized by a single isolate are not represented in the figure. The isolates were categorized according to sample type into raw meat (RM), food processing environment (FPE), and end product. The *Leuc. carnosum* and *Lb. sakei* PFGE types were color-coded representing different biochemical fermentation profiles and carbohydrate utilization patterns. The numbers on the right represent the percentage of carbohydrates utilized per isolate, while the numbers on the bottom represent the percentage of a single carbohydrate per studied group of isolates. Gradient scale: 0% (red)—100% (green). Abbreviations: B: brine; BC: brine cultures; CB: cutting board; CH: cooked ham G: gloves during packaging of the end product; HAT: ham after tumbling; CC: cellulose casing; RM: raw meat; RMDB: raw meat delivery box; T: trolley; TAS: tumbler after sanitation.

All *Lb. sakei* isolates (n = 48) were found to utilize five carbohydrates, namely D-glucose, D-fructose, N-acetyl glucosamine, D-mannose, and D-galactose (Figure 3b). Most of the *Lb. sakei* isolates utilized D-ribose, L-arabinose, D-sucrose, D-trehalose, gentibiose, D-cellobiose, D-melibiose, gluconate, and esculin. However, D-xylose, D-turanose, D-maltose, D-lactose, salicin, amygdalin, and arbutin were utilized specifically by certain isolates. Based on the carbohydrate utilization patterns of the tested *Lb. sakei* PFGE types, they were categorized into 14 distinct BP. Two PFGE types (Sakei 7 and Sakei 10) that shared BP 7 utilized 26.5% of carbohydrates when isolated from raw meat, FPE, and cooked ham. Other PFGE types (Sakei 3 and Sakei 4) isolated from raw meat, FPE, and cooked ham displayed slight differences in carbohydrate utilization percentages. The PFGE type Sakei 4 carbohydrate utilization ranged between 22.4% (BP 1), 24.5% (BP 3), and 26.5% (BP 8). The PFGE type Sakei 3 was represented by BP 6 and BP 5 when isolated from the raw meat, FPE, and cooked ham. The BP6 utilized 24.5% of carbohydrates, while BP 5 lacked salicin utilization and fermented 22.4% of carbohydrates. The most common biochemical patterns in cooked ham were BP 3 and BP 5, while sporadically isolated PFGE types (Sakei 6, Sakei 1, and Sakei 5) in cooked ham utilized 26.5% (BP9), 28.6% (BP10 and BP11) of carbohydrates. The same biochemical profile was not shared by different PFGE types in cooked ham, as observed with *Leuc. carnosum* PFGE types. We observed no difference in carbohydrate utilization among isolates with identical PFGE types that were isolated at the beginning, during, and at the end of cooked ham storage. The highest utilization ability of 32.7% carbohydrates (BP 14) was observed for PFGE type Sakei 9, isolated from brine (BP 14). Additionally, other PFGE types from FPE, including Sakei 4-ST, Sakei 11, and Sakei 8, isolated from brine, tumbler after sanitation, and cutting board, utilized 22.4% (BP 2), 28.6% (BP 12), and 30.6% (BP 13) carbohydrates, respectively.

The *Leuc. carnosum* PFGE types had positive acid and alkaline phosphatase enzymatic activity (Table S5). Furthermore, naphthol-AS-BI-phosphohydrolase activity was present in all PFGE types except for Leuc 11 and Leuc 12. The *Lb. sakei* PFGE types had positive leucine and valine arylamidase enzymatic activity. The highest enzymatic activity was observed in PFGE type Sakei 11, identified after tumbler sanitation (Table S6).

4. Discussion

The marketing of ready-to-eat convenience products, such as cooked ham, is increasing due to consumer demand for less processed foods and high organoleptic quality [1,30,31]. However, cooked ham is a perishable meat product with pH values ranging from 5.5 to 6.5, water activity (aw) between 0.95 and 0.99, and readily available nutrients such as glucose, ribose, amino acids, and nucleosides. These characteristics make it an ideal growth medium for a wide range of microorganisms originating from raw meat, other ingredients, or the food processing environment (FPE), which can potentially compromise the safety and shelf life of the final product [10,32]. Vacuum packaging and cold storage are commonly used to prevent the growth of spoilage microorganisms. However, these measures often result in

selective pressure towards psychrotrophic and strictly or facultatively anaerobic microbes, such as LAB [10].

We used a maximum acceptable limit of 7.4 $\log_{10}$ AMC/LAB CFU/g for the end of shelf life in cooked ham, in accordance with the AMA quality seal requirements in Austria [21]. The aim of the study was the determination of the timepoint when the maximum acceptable limit during cold storage of cooked ham was exceeded. Other questions included the effect of the microbial load and composition of the raw meat on the microbial limit of the cooked ham and the effect of the hygiene conditions in the pre- and post-cooking areas of the FPE on the exceeding of the shelf life of the cooked ham. The microbial growth dynamics in nine different lots of cooked ham varied, with initially low LAB populations. The LAB population count in cooked ham exceeded the maximum acceptable microbial limit on different days, with a minimum of 11 and a maximum of 20 days of storage. Even when the raw meat was treated with brine cultures to extend the shelf life of the cooked ham, the maximum acceptable limit was exceeded during storage of the cooked ham on days 11 and 20. These observations are not surprising, as the shelf life of vacuum-packed or MAP-packed processed meat products, including cooked ham, is generally dictated by LAB growth [1,10]. Moreover, previous studies also reported rapid LAB growth from initially low LAB levels in freshly packed cooked ham [5,30,33]. The intrinsic characteristics of cooked ham, such as water activity, sugar-enriched preparations, and the presence of sodium chloride and sodium nitrite, provided a selective advantage for the growth of strict and facultative anaerobic LAB [7,8,12,30]. The observation of a plateau in LAB growth when the limit of 7.4 log10 CFU/g was exceeded could be attributed to acid production and nutrient depletion, as previously reported when LAB counts reached 8.0 or 9.0 $\log_{10}$ CFU/g [5,33]. Several other studies observed LAB growth above 7.0 $\log_{10}$ CFU/g in cooked ham also reported acid flavor, slime and gas production, off-odors, and cooked ham discoloration [5,18,34]. However, in contrast to these studies, we did not observe any sensory alterations in cooked ham, except for the decrease in pH, which was only measured during the storage of lots 8 and 9. These observations suggest that the spoilage process is not solely caused by the microbial count but also by the accumulation of metabolic byproducts from specific microorganisms [35]. Although other LAB bacteria (*L. mesenteroides*, *Carnobacterium divergens*, *Leuconostoc gelidum* subsp. *gasicomitatum*) and non-LAB bacteria (*Pseudomonas*, *Kocuria*, *Corynebacterium*, *Bacillus*, *Staphylococcus*, *Enterococcus*) were isolated at the beginning of storage, they were outcompeted by the rapid growth of *Leuc. carnosum* and *Lb. sakei.* However, a slimy surface texture was observed during storage of cooked ham lot 9. The observed spoilage effects could be attributed to the isolation of spoilage organisms, such as *L. gelidum* subsp. *gasicomitatum*, *L. curvatus*, *C. divergens*, which are known to form slime and produce acidic or buttery off-flavors [10]. Both species were identified as part of a raw meat psychrotrophic microbiota identified in meat during the tumbling process, following their isolation in cooked ham [12]. It was surprising to observe the absence of microbial growth during the 20 days of storage of cooked ham lot 6. This observation is consistent with the results reported by Zagdoun et al. 2021 [9], where AMC and LAB counts showed a lag phase around 22 and 28 days and an exponential phase around 40 days of storage. The absence of microbial growth observed during the storage of lot 6 may be explained by the limited selectivity of the ISO method, particularly with regard to psychotropic bacteria, which are typically underestimated [9,13]. Consistent with our findings, Zagdoun et al. [9] also reported fluctuations in LAB counts during the storage period, along with variability in the initial concentration at the beginning of the storage. This suggests that the observed variability in LAB growth among different lots of cooked ham may be attributed to cross-contamination events occurring under the conditions of the processing facility [9].

In determining the final microbiota of cooked ham, the raw meat, the processing environment, and the hygiene conditions throughout the processing line play a crucial role. In addition to investigating the AMC/LAB level during cooked ham storage, our study focused on evaluating the microbial load and composition of the raw meat input, the

hygiene conditions in the FPE, and in particular, the isolation of LAB, which dominates under psychrotrophic conditions during cooked ham storage.

Therefore, after assessing the initial microbial status of the three cooked ham lots, we also conducted microbiological investigation of the raw material in subsequent production lots. We did not observe any association between the microbial load of the raw meat and how fast the microbial limit was exceeded during cooked ham storage due to the small amount of analyzed raw meat samples. A limitation of the study is the small number of raw meat samples analyzed. However, the isolation of LAB from raw meat and FPE and subsequent identification of the isolates by molecular subtyping confirmed the contamination of the final product by raw meat. Samelis et al. [20] reported a 20% presence of typical *L. carnosum* strains on raw pork meat used for cooked ham production. Other studies have reported recontamination of products in the post-cooking area prior to packaging with about 0.5–2.0 $\log_{10}$ CFU/g of mainly LAB [18]. The characterization of the isolates by molecular typing indicated that the contamination of the final product originated from the raw meat and the post-cooking area. The most frequently isolated strains included *Leuconostoc carnosum* (Leuc) PFGE types Leuc 2, Leuc 3, and *Latilactobacillus sakei* (Sakei) PFGE types Sakei 3 and Sakei 4, which were both present in raw meat and the post-cooking area of the FPE. Prior studies have addressed the impact of raw meat and FPE on the composition of the final microbiota in cooked ham mainly by 16S rRNA gene profiling and by culture-dependent methods [9,11,12,16,19,20]. However, the present study provides confirmation of these observations at the strain level.

In eight out of nine cooked ham lots tested, there was no microbial growth at the beginning of storage (day 0), indicating appropriate hygienic and processing conditions, which were supported by the low microbial counts observed on the surfaces in the pre- and post-cooking areas of the FPE as proposed by Garriga et al. [30]. These observations indicated that even if the microbial count in FPE is low, recontamination of the end product with a small fraction of specific LAB strains can reduce the shelf life of cooked ham. In fact, both LAB species, *Leuc. carnosum* and *Lb. sakei*, were identified in the tumbler after disinfection. The meat production plant uses a combined alkaline detergent-sanitizer (DS), which can eliminate most microorganisms. However, it has been shown that this type of disinfectant is not sufficient to remove LAB [20]. Furthermore, the absence of LAB growth at the start of storage can be attributed to sublethal damage during the cooking step at 72 °C for 60 min. However, with prolonged storage, the sublethally damaged LAB resuscitated, leading to the microbial limit being exceeded. Veselá et al. [12] demonstrated a similar phenomenon, where sublethally damaged LAB were able to resuscitate after cooking at a core temperature of 72 °C for 10 min during storage of cooked ham.

Previous studies have reported variability in biochemical characteristics among *Leuconostoc* sp. and *Latilactobacillus* sp. strains isolated from various sources, including dairy-related products, modified atmosphere packaged sausage, and cooked ham [10,36,37]. These findings align with our observations. In addition, biochemical characterization of frequently isolated strains from cooked ham, raw meat, and FPE revealed specific adaptations in carbohydrate utilization, reflecting their adaptation to the source of isolation. For instance, the PFGE type Leuc 2 from raw meat indicated a distinct carbohydrate utilization pattern by fermenting D-mannose, D-galactose, D-maltose, D-melezitose, and D-raffinose, which was not observed among the isolates from cooked ham. Similarly, *Lb. sakei* PFGE types Sakei 3 and Sakei 4 from raw meat, FPE, and cooked ham showed slight differences in carbohydrate utilization patterns, including D-melibiose and salicin, depending on the isolation source. These observations emphasize the different fermentative capabilities among the strains of the same species. Therefore, the growth capabilities and dynamics in the meat matrix, competitiveness against other bacteria, and production of spoilage-related molecules of *Leuc. carnosum* and *Lb. sakei* strains identified in the present study need to be investigated [10]. Some *Leuc. carnosum* and *Lb. sakei* originating from unspoiled samples have already been considered potential bioprotective cultures in meat-based products in other studies [1,20,38,39].

Overall, the data demonstrate that the presence of LAB in raw meat and FPE influenced the exceeding of the microbial limit during the cooked ham storage without causing sensory defects. Our observations are in line with the recent observations of Alessandria et al. [1], who also highlighted the discrepancy between microbial counts and sensory defects in cooked ham at the end of shelf life. They observed that some cooked hams with higher microbial counts did not exhibit sensory defects. Furthermore, some samples showed similar independent microbial counts in spoiled and unspoiled cooked ham samples. Similarly, the microbiota analysis conducted by Raimondi et al. [9] in the cooked ham at the end of its shelf life did not identify any specific taxon association with cooked ham spoilage. These findings suggest that the spoilage effects may be linked to specific strain characteristics within the same species or possibly to subdominant bacterial groups.

The present study emphasized the challenges of controlling LAB growth in vacuum-packed cooked ham despite high hygiene standards and processing conditions. In order to ensure food safety, it is important to consider that the presence of low LAB numbers in raw meat and FPE can limit the shelf life. Furthermore, future research should focus on understanding the spoilage potential of specific strains of LAB that are associated with sensory alterations in cooked ham. Instead of relying solely on a strict limit of 7.4 $\log_{10}$ CFU/g to determine the end of shelf life, it is important to consider the specific characteristics and behaviors of LAB strains that contribute to spoilage in cooked ham.

5. Conclusions

There is an increasing consumer demand for cooked ham with an extended shelf life. The presence of lactic acid bacteria during cooked ham storage has a controversial role due to discrepancies between the maximum acceptable microbial load and sensory deficiencies during cooked ham storage. The analysis of a large number of samples during the storage of cooked ham demonstrated that lactic acid bacteria, *Leuc. carnosum* and *Lb. sakei*, were responsible for exceeding the microbial limit for the end of shelf life without observed sensory deficiencies after 20 and 33 days of storage. Furthermore, the presence of the most frequently identified *Leuc. carnosum* PGFE types Leuc 2 and *Lb. sakei* PFGE types Sakei 3 and Sakei 4 in cooked ham was associated with raw meat and recontamination events in the post-cooking area of the food processing environment, including the unwrapping and cutting of cooked ham. While controlling the growth of psychrotrophic facultative strict and facultative anaerobic lactic acid bacteria in cooked ham remains a challenge, it may be advisable to focus on the identification of specific spoilage organisms instead of relying solely on a strict microbial limit to determine the end of shelf life in the cooked ham.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12132475/s1, Table S1: Overview of cooked ham processing steps; Table S2: Overview of isolates recovered from raw meat, food processing environment, and cooked ham; Table S3: Isolates identified in raw meat, food processing environments, and cooked ham by partial sequencing of bacteria's 16S rRNA gene and the internal transcribed spacer 2 (ITS2) region for fungi; Table S4: Description of isolates identified in cooked ham during the storage of lots 8 and 9 with brine cultures; Table S5: Identification of enzymatic activity in *Leuconostoc carnosum* strains; Table S6: Identification of enzymatic activity in *Latilactobacillus sakei* strains.

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4 DISCUSSION

4.1 Summary of the results

The thesis aimed to address microbial contamination challenges in primary poultry production and post-harvest processing of pork meat, triggered by ongoing discussions with meat production facilities in Austria. This research aimed to fill data gaps hindering advanced risk assessment during meat processing, focusing on *Campylobacter* spp. presence in poultry drinking water and the microbial quality of RTE cooked ham pork product. Selected production steps were tailored to the specific microbial safety and quality challenges during meat production, with overall goal of enhancing GMP and HACCP conditions. The methodology involved culture-based methods and partial sequencing of 16S rRNA to identify and characterize microbiota in both primary poultry production and pork meat processing environment. Results revealed a high microbial load and diversity in at the end of drinking water before and after waterline sanitation in poultry farms in Austria, with *Campylobacter jejuni* detected in a single sample. Furthermore, the study evaluated the shelf-life of vacuum-packed cooked ham, identifying *Leuconostoc carnosum* and *Latilactobacillus sakei* as the primary contributors to exceeding the shelf-life limit during refrigerated storage. While the investigations were focused on characterizing microbial contamination scenarios during meat production and processing in Austrian production facilities, adhering to Austrian and European food safety and quality regulatory standards, the findings from this study have broader implications. The results from the present study can be extrapolated to diverse food production environments to increase our knowledge about the food processing microbiota. Furthermore, the results generated in the present study made significant impact in educating producing poultry farms in improved hygiene strategies, as well as for training the employees in the meat processing facility for improved GMP and HACCP condition during pork meat processing.

4.2 Assessment of meat safety and quality using culture-based methods

The use of culture-based methods allowed for the assessment of microbial contamination in accordance to regulatory standards. These methods covered AMC, EB, LAB, and PS counts as indicators of microbial quality of poultry drinking water, assessing cooked ham meat quality, and monitoring hygiene throughout the cooked ham processing. In both studies the contamination assessment was based on prior research recommendations and quality certifications such as Agrar Markt Austria Marketing GmbH (AMA) quality seal label criteria [106–108].

4.2.1 Implications of culture-based methods for safety control during poultry primary production

Rapid and accurate detection of foodborne pathogens and hygiene indicators are essential for food safety and quality in prevention of foodborne infections [109]. Currently, standardized methods, such as ISO methods, are recognized as the official reference analytical methods for quality and control [85]. The ISO methods have been developed for pathogens and spoilage organisms, focusing on the presence or absence and counts of these microorganisms, to meet legal and regulatory food quality and safety standards [86]. The main advantage of these methods is that they have been optimized over the years, are cost-effective, assess viable microbes in a sample, easy to quantify in a sample, and achieve a high sensitivity with selective media. Despite their established use, culture-based methods, while recognized internationally, have limitations. They often fail to detect minor microorganism populations under controlled conditions (i.e. selective media, incubation time and temperature, and oxygen availability) and can overlook pathogens existing as viable but non-culturable cells (VBNC) [5,26,110]. This limitation became evident during the assessment of *Campylobacter* spp. presence in poultry drinking water. Microorganisms exposed to chemical or physical processes that do not kill them may exist in VBNC or a sub-lethally damaged state [111].

Campylobacter spp. are known to exist in low numbers in drinking water and can also be present in a VBNC state [112]. In the VBNC state, bacteria are alive but remain undetectable using standard culture media [113]. Consequently, due to the low sensitivity of culture-based methods in detecting bacteria in low numbers and/or in the VBNC state, it is possible that *Campylobacter* spp. might have been overlooked during the investigations.

4.2.2 Implications of culture-based methods for quality control during cooked ham storage

When exposed to technological processes like cooking, bacteria may enter a reversible sub-lethally damaged state [114]. Although, *Leuconostoc (Leuc.) carnosum* and *Latilactobacillus (Lb.) sakei* were occasionally isolated from raw meat due to their low presence in raw meat, these bacteria were undetectable in cooked ham after the cooking step and up to four days post-production. Sub-lethally damaged bacteria present challenges in food diagnostics when utilizing culture-based methods for detection and enumeration [115–117]. Conventional culture media, containing selective compounds potentially harmful to injured cells, may lead to the underestimation of survivor levels, resulting in false negatives [111]. Stressed cells responding with specific reparative processes may exhibit a longer lag phase compared to healthy cells, often falling below the detection threshold of culture-based methods [118]. However, given sufficient recovery time, as observed during cooked ham storage, complete recovery of cell activity is possible [111]. This recovery was observed by exponential growth during the cooked ham shelf-life after the sixth day of storage.

4.2.3 Next generation sequencing in meat microbiology: advantages and limitations

The culture-based techniques are inherently biased, time-consuming, and involve multiple labor-intensive steps for microbial isolation, which restrict their capacity to provide a comprehensive understanding of microbial communities [5]. To complement these traditional methods, culture-independent molecular techniques, such as NGS, have emerged and transformed food microbiology. NGS methods offer rapid and in-depth analysis of microbial diversity [26,119–121]. In addition, the growing adoption of NGS methods is driven by the technological and sequencing advancements, cost reduction, and its widespread application in diagnostics, outbreak investigations, antimicrobial resistance studies, and food authenticity verification [122]. NGS methods in food microbiology aim to identify both dominant and sub-dominant bacterial populations and assess how spoilage affects microbial communities [123,124]. It is a valuable tool for understanding dynamics of microbial population during different storage conditions or processes [119]. However, limitations of NGS methods include presence of bacterial pathogens in food and FPE in low numbers or low proportions relative to other species, which may hinder their detection [125]. In addition, NGS methods cannot distinguish viable from non-viable cells [126]. The NGS methods present challenges for food regulators, particularly in determining absolute pathogen abundance. Establishing limits of detection for low-level pathogens and addressing sequencing technology's misclassification are regulatory challenges that demand standardized methods for pathogen detection and quantification [125,127,128]. Standardizing sample preparation, sequencing, and data analysis minimizes errors and bias, ensuring the quality and comparability of data [122,123].

4.2.4 Assessment of meat safety and quality: Combined culture-based detection with molecular identification

The utilization of culture-based methods in combination with partial sequencing of 16S rRNA gene in the present research thesis enabled identification and assessment of diversity among major bacterial contaminants. Partial sequencing of the 16S rRNA gene has gained widespread use in bacterial taxonomy identification due to its advantages over phenotypic methods [129]. Despite utilizing a discrimination level of ≥99% for species for species identification, differentiation between the majority of identified *Pseudomonas, Enterobacteriaceae*, and *Bacillus* species in poultry drinking water was not possible, necessitating classification at the genus level. This observation aligns with previous reports [130–132]. The challenge in species identification originates from limited variations in 16S rRNA gene sequences among closely related taxa, attributed to its conserved nature [133]. While prior studies encountered difficulties in distinguishing *C. jejuni, C. coli*, and *C. lari* using 16S rDNA, our research successfully identified *C. jejuni* via 16S rDNA sequencing [134]. This identification was supported by *Campylobacter*-specific multiplex PCR, as demonstrated by Wang et al. in 2002 [94]. Additionally, LAB species were identified at the species level with 16S rDNA sequencing, exhibiting ≥99% sequence identity, subsequently confirmed by molecular-typing methods and biochemical identification (API 50 CHL). Further limitations of isolate identification using partial 16S rRNA gene sequencing include the necessity for high sequence quality, lack of consensus on the percentage similarity required to assign sequences to a specific species or genus (ranging from 97% for genus to 99% for species level), and reliance on databases for sequence [95,129].

While culture-based methods come with inherent biases, they remained the most direct approach for evaluating drinking water safety and cooked ham quality at both poultry farms and a meat processing facility in Austria. Moreover, regardless the limitations associated with partial 16S rDNA sequencing, this method enabled the identification of key bacteria crucial to meat safety and quality conditions, offering a rapid,

straightforward, and cost-effective solution. While culture-based methods may lack the specificity and sensitivity of culture-independent and NGS methods, the outcomes derived from investigations conducted at poultry farms and meat processing facilities validated their proficiency in evaluating contamination levels, identifying production-related concerns, and tracing contamination transmission pathways. They do so without requiring complex sample preparation, analysis, or high costs. Furthermore, the data generated through these methods can be further explored using advanced NGS techniques, like whole genome sequencing (WGS), to gain deeper insights into the relationships between the isolates collected from the meat processing environment. Lastly, culture-based methods remain vital for ensuring food safety and quality in food production industry due to their reliability, efficiency, and international standardization. Therefore, the application of culture-based methods was necessary in this study, as it aimed to assess the compliance of food production and safety practices in accordance with both national and international food safety and quality legislation. As a result, the comparison between findings from the cooked ham storage and the national AMA quality certificate seal became feasible. Furthermore, these methods facilitated the assessment of poultry drinking water quality, allowing for comparative analysis with other studies conducted in this field.

4.3 Microbial safety assessment in poultry primary production

4.3.1 *Campylobacter* spp. identification before and after drinking waterline sanitation

Farm-level biosecurity provides the foundation for biosecurity across the entire meat production chain, especially in the EU, where the production strategy is based on safety throughout the entire meat production chain [135,136]. The investigations conducted at poultry farms aimed to bridge the knowledge gap concerning the effectiveness of water management practices within farm-level biosecurity. Specifically, the study focused on

identification of *Campylobacter* spp. presence in drinking water before and after drinking waterline sanitation at poultry farms in Austria. Contaminated drinking water has been identified as a potential source of *Campylobacter* spp. on poultry farms [137]. Hence, without proper waterline sanitation, drinking water may serve as a transmission vehicle for *Campylobacter* spp. among poultry flock [62,138,139].

The direct confirmation of *Campylobacter* spp. in drinking water samples at poultry farms in Austria was not established in majority of tested samples due to the previously mentioned possible limitations of culture-based methods as an enrichment step is mandatory. Drinking water samples with high microbial levels may contain a significant amount of non-targeted microbial groups that may out-compete the growth of *Campylobacter* spp. during the enrichment process [140]. We employed Bolton broth for selective enrichment and isolation of *Campylobacter* spp. from water samples, following ISO 10272-1:2006. Previous studies have suggested that sensitive detection of *Campylobacter* spp. may require different water concentration techniques and processing larger volumes of water [141]. However, in our study, we opted for a simpler approach, utilizing 500 mL water sample centrifugation at 8000 rpm. *Campylobacter* spp. are typically found in water in relatively small numbers [142,143]. Thus, another potential reason for the non-detection of *Campylobacter* spp. in the majority of water samples could be attributed to the absence of sample concentration. This process involves filtration of water samples of varying volumes (1000 ml, 100 ml, and 10 ml) using 0.45 µm and/or 0.20 µm pore-sized membrane filters, followed by placement of the filters into enrichment broth [141]. It was demonstrated that filtering water volumes from 1 to 3 liters increases the number of *Campylobacter* spp. positive samples [8]. However, the results indicated high prevalence of opportunistic human and animal pathogens, such as *Pseudomonas* spp. and *Stenotrophomonas* spp., in the majority of water samples. These findings highlighted inadequate waterline management practices, as the frequently identified bacteria are known for their ability to form biofilms [107].

Previous investigations have shown prolonged *Campylobacter* spp. survival in drinking water in association with biofilms [144,145].

Current research strategies primarily focus on monitoring strategies and reactive interventions regarding *Campylobacter* spp. risks associated with poultry farms [63]. This is due to the complexities of *Campylobacter* spp. transmission cycles in poultry farms and the limited understanding of underlying mechanisms [146,147]. Multiple sources contribute to *Campylobacter* spp. presence in poultry farms, including the environment, humans, farm utensils, equipment, water sources, and other chicken flocks [136,148–150]. These sources are variable and seasonal, leading to undetected infections as poultry typically do not display symptoms [151]. Initially, we hypothesized that poultry drinking water could be a significant factor in *Campylobacter* spp. colonization during the fattening phase. However, the potential underestimation of *Campylobacter* spp. at Austrian poultry farms might be attributed to the lack of water sample filtration and the potential presence of *Campylobacter* spp. in the VBNC state. As a result, it remains inconclusive whether poultry drinking water presents a risk of *Campylobacter* spp. infection within the flock.

4.3.2 Importance of drinking waterline sanitation during poultry production

The poultry farms involved in the study implemented waterline sanitation protocols during production intervals, employing either 4 ppm ClO_2 or a combination of chemicals (4 ClO_2, 3% peroxyacetic acid (PAA)) along with mechanical waterline treatment using an air-pressure pump. The assessment of poultry drinking water resulted in a microbial load exceeding 4 log CFU/ml across the majority of poultry farms in Austria, both before and after waterline cleaning procedures. This suggests that employing mechanical cleaning alongside higher concentrations of sanitizing agents might offer enhanced efficacy compared to the currently applied concentrations of 4 ppm ClO_2 and 3% PAA.

However, existing research lacks comprehensive studies on the optimal concentrations required for effectively reducing high microbial loads in poultry drinking water, as previously noted [152]. The current biosecurity measures, which involve waterline treatments between flocks, are established on informal guidelines for poultry farmers regarding the use of chlorine dioxide, hydrogen peroxide, acetic acid, and peracetic acid [153,154]. Nonetheless, the ineffectiveness of these informal guidelines, particularly concerning the recommended concentrations applied during waterline treatments, becomes apparent based on observed high microbial loads in water samples. Furthermore, the assessment of poultry drinking water at poultry farms highlights the significance of accurately determining the optimal concentrations of sanitizing agents to ensure effectiveness. Recommended concentrations of cleaning and disinfection agents may not always be sufficiently high to effectively decontaminate waterlines. Notably, a single study conducted on a poultry farm in the United States utilized a concentrated chlorine-based disinfectant with over 1000 ppm of free chlorine residual for cleaning waterlines between flocks [106]. This, along with daily sanitation using 0.5 to 1 ppm free chlorine residual, effectively maintained the microbial load in water below 3 log CFU/ml.

Moreover, variations in microbial loads were observed at different times in poultry drinking water within the sampled farms that were assessed on multiple occasions. This variability highlights inconsistencies in poultry farm practices and suggests a potential lack of awareness among farmers regarding the importance of microbial water quality for both animal and human health. A study on poultry farms in France was in line with the observations from the present thesis by revealing that at more than 50% of the analyzed farms (n=1004) exhibited considerable room for improvement in cleaning and disinfection practices at commercial intensive poultry farms [155]. The elevated microbial load was consistently associated with the frequent isolation of opportunistic pathogens including *Pseudomonas* spp., *Stenotrophomonas* spp., and *Ochrobactrum* spp., exhibiting resistance patterns to antibiotics commonly utilized in poultry

production. While microbiological contamination levels are defined for water quality at the source, no regulations or guidelines are defined for water quality at the end of the drinking line. This gap likely poses potential risk for food safety, quality, and to animal and human health. Therefore, this study confirmed the hypothesis regarding poultry drinking water as a possible risk to safety and quality at poultry production farms, likely linked to inadequate waterline management.

4.4 Assessment of microbial quality in processed cooked ham

4.4.1 Lactic acid bacteria originating from raw meat and processing environment: A major limitation of cooked ham shelf-life

The quality of cooked ham relies on both the quality of the raw materials and the processing steps, such as brine injection, tumbling, and cooking at temperatures exceeding 72°C [35,156,157]. Cooked ham contamination during its shelf-life typically arises from LAB present in the raw materials and/or introduced during the processing stages [158]. The findings from this study align with previous investigations, confirming that LAB were the primary bacteria responsible for exceeding 7.4 log CFU/g shelf-life limit in vacuum-packed cooked ham.

Earlier studies have utilized NGS methods to explore the microbial ecosystem of cooked ham during processing stages [157]. However, this study provided deeper insight by identifying prevalent bacterial strains at a strain level within the processing environment and during the storage of cooked ham. The identification outcomes of the most prevalent *Leuc. carnosum* and *Lb. sakei* strains in cooked ham, using the molecular typing PFGE method, were compared with strains found in raw meat and the processing environment. This comparison revealed the presence of *Leuc. carnosum* PFGE types Leuc 2, and *Lb. sakei* PFGE types Sakei 3 and Sakei 4 in cooked ham, raw meat, and the post-cooking area of the ham processing environment. These results confirmed the hypothesis indicating that the primary introduction of LAB into the processing environment

stemmed from the raw materials. Subsequently, potential re-contamination event in the post-cooking area were observed, possibly facilitated by workers gloves and food contact surfaces.

The investigated cooked ham processing facility reported several non-standardized processing steps in the production facility, potentially contributing to the observed short shelf-life of vacuum-packed cooked ham. These practices involved storing raw materials in cold rooms for up to five days and employing prolonged tumbling times. This resulted in identification of different *Leuc. carnosum* and *Lb. sakei* PFGE types in raw meat, brine, and ham after tumbling. Thus, the extended storage of raw meat in cold rooms and the use of longer tumbling times might have facilitated increased bacterial growth and allowed bacteria to penetrate deeper tissues, possibly offering protection against high cooking temperatures. Consequently, the results showed onset of LAB growth between storage days four and six. This growth phase possibly indicates the time required for sub-lethally damaged bacteria to resuscitate, transitioning into a logarithmic growth phase which was observed between cooked ham storage days nine and twenty. This observation aligns with previous studies that indicated the cooking step might not consistently eliminate thermotolerant LAB [159]. Hence, the findings from cooked ham storage emphasize the critical need for precise core temperature assessment during cooking [160]. Moreover, further investigation is necessary to implement improved cooking practices without altering the cooked ham sensory characteristics.

The investigation of tumbler after sanitation resulted the presence of *Leuc. carnosum* and *Lb. sakei* PFGE types, distinct from those identified during cooked ham storage. Indeed, previous observations have indicated that sanitation measures may not always effectively eliminate LAB contamination [81]. In the food industry, detergents and sanitizers are utilized either separately or in combination [161]. At the investigated cooked ham processing facility, detergent-sanitizer (DS) products were utilized to clean both the tumbler and the brine injector. Furthermore, an alkaline detergent was applied weekly to clean the trolleys used in the cooling process for cooked ham. However,

previous investigations recommended separate use of detergents and sanitizers for optimal antimicrobial efficacy [162]. The study by Mäkelä et al. [141] demonstrated that DS products containing multiple antimicrobial compounds were less effective against ropy-slime producers compared to individual sanitizers. This reduced efficacy of DS products was attributed to the presence of surface-active compounds, which can alter the antimicrobial properties of the product. Investigations within the processing environment confirmed the presence of *Leuc. carnosum* PFGE types on trolleys, indicating that cleaning the trolleys once a week might not be sufficient for ensuring adequate hygiene.

4.4.2 Shelf-life assessment: Evaluation of maximum acceptable microbial limit during cooked ham storage

The monitoring of microbial growth during cooked ham storage revealed a discrepancy between the observed maximum acceptable limit for the end of shelf-life and sensory defects. Shelf-life limitations generally result in rapid deterioration of the sensory qualities of cooked ham, i.e. appearance, taste, odor, tenderness, hardness, springiness, cohesiveness, gumminess, chewiness, juiciness, and color [81]. Throughout the investigations, no sensory alterations were noted in the cooked ham, except for a decrease in pH, which was analyzed during the storage of 2 out of 9 cooked ham lots. The present work defined the shelf-life limit as a maximum acceptable count of 7.4 log CFU/g, in line with Austria quality standard label [108]. The limit is determined collectively by various stakeholders, including regulatory bodies and consumer requirements. It serves as a guide for the manufacturing process, facilitating a comprehensive evaluation of product quality and the definition of preventive actions [108]. However, this limit did not necessarily correlate with observable sensorial alterations. During the microbiological investigations of cooked ham shelf-life, no change of color, off odors were observed. Moreover, maximum acceptable microbial levels in cooked ham products vary widely in the literature, ranging from 6 log CFU/g

to 9 log CFU/g [81,163]. This variability suggests that microbial growth rates and final concentrations alone may not determine the end of shelf-life. Metabolic activities during growth phase, likely play a crucial role in determining shelf-life limitations and product spoilage, an aspect not explored in this study [164]. However, defining metabolic quality indicators as a spoilage biomarkers, remains challenging due to limited nutrient-based indicators and the diverse range of potential end metabolites [81].

Perceived spoilage, attributed to the microbial production of various metabolites like organic acids, carbon dioxide, amines, sulfur compounds, dextran compounds, alcohols, aldehydes, and ketones, is commonly associated with multiple external factors [43,156]. These factors include overall processing methods, storage temperature, packaging conditions, interactions among the bacteria, and inherent properties specific to cooked ham products.

The storage of cooked ham revealed the predominance of two primary LAB species, namely *Leuc. carnosum* and *Lb. sakei*. However, the association of limited number of LAB with cooked ham spoilage suggests a high diversity at the species level, emphasizing the importance of considering diversity on strain level. Moreover, certain LAB strains possess attributes like the production of organic acids, competitive exclusion, quorum sensing, or antimicrobial metabolites like bacteriocins, which can inhibit the growth of foodborne pathogens or spoilage bacteria [165–167]. An investigation into these characteristics among the frequently isolated strains during present thesis using WGS and phenotypic tests holds the potential to significantly advance our understanding of their protective or spoilage traits. Such an investigation could offer valuable insights into their potential application as starter cultures within the meat industry. Improving safety and quality measures in poultry and pork meat production.

4.5 Improving safety and quality measures in poultry and pork meat production environment

Training employees to recognize abnormal conditions, designing specific plans to investigate potential hygiene deficiency niches, and considering different product proximity for contamination spread are essential. In present thesis, inadequate behavior combined with inadequate cleaning and sanitation practices due to insufficient knowledge were observed in both poultry farms and cooked ham production facilities. As a result, the microbiological assessments conducted at these sites were shared with poultry farmers and the quality assurance manager in the meat processing facility, aimed at enhancing their understanding and practices regarding biosecurity and hygiene management.

Although conducted studies did not directly implement or test specific interventions at poultry production farms and processing facilities, the findings offer valuable guidelines. For poultry farms, these guidelines involve the implementation of routine waterline mechanical treatments, evaluating the effectiveness of current waterline sanitation solutions, and potentially adjusting their concentrations. Alternatively, specialized contractors in waterline management could be considered.

Similarly, insights gained during the investigations of cooked ham processing can serve meat processing facilities in optimization of HACCP procedures regarding raw material storage, cleaning practices, and post-cooking area handling procedures. This optimization includes fast processing of delivered raw meat, separate application of detergents and sanitizers, accurate monitoring of core cooking temperatures, and comprehensive hygiene training for employees in pre- and post-cooking areas.

The food production focus is shifting from mass production to high-value products, prioritizing quality, safety, and compliance at every stage [168]. The increasing demand for poultry and pork meat and their products has elevated bacterial-related challenges during meat processing. Therefore, continuous efforts are being made to enhance

monitoring and risk assessment, yet a conclusive solution is still missing. This thesis has provided insight into the complexity of these problems and emphasized crucial aspects concerning the quality and safety of poultry and pork during the specific production steps along the production chain. The outcomes of these investigations offer valuable insights for poultry farmers and cooked ham production facilities, enabling the identification of hygiene risk areas and providing guidelines for improvement.

5 FUTURE DIRECTIONS

Addressing safety risks during primary production and maintaining strict hygiene standards in meat processing are crucial for effective mitigation strategies. This thesis provided initial insights into the microbial quality of poultry drinking water and highlighted LAB transmission routes during cooked ham processing, affecting the product shelf-life. Yet, additional actions should be taken to improve meat safety and quality at poultry farms and in meat processing environments.

Future investigations to ensure safe meat production, particularly in poultry settings, should include waterline management strategies. This includes comprehensive testing of water quality from the source to the end of the drinking line. Assessing appropriate concentrations of sanitizing and cleaning agents based on initial microbiological concentrations in drinking lines is crucial. For identifying drinking water as a source of *Campylobacter* spp., analyzing water samples should involve filtration, potential pre-enrichment methods, and employing culture-independent techniques in combination with cell viability assays. Enhancing the sensitivity of detection methods for *Campylobacter* spp. in poultry drinking water would deepen our understanding of *Campylobacter* spp. ecology within poultry farms, ultimately improving animal hygiene and reducing the risk for contamination during slaughter and meat processing.

Concerning cooked ham shelf-life, future investigations should focus on observing microbial growth while monitoring fermentation indicators like pH and metabolites (such as ethanol, D- and l-lactate, acetate, and formate). Analyzing the identified LAB strains using WGS could provide insights into genetic diversity, phage presence, metabolic pathways, bacteriocin production, and antibiotic resistances. However, complementing WGS findings with phenotypic tests, such as co-culturing with known spoilage LAB and pathogens like *L. monocytogenes*, and evaluating strain antimicrobial effects in cell-free supernatants is essential. Furthermore, investigating strain resistance to cooking temperatures, both in vitro and within the meat matrix, is crucial for a

comprehensive understanding of these LAB strains. Therefore, conducting a comprehensive investigation into the identified strains during cooked ham processing contributes to enhancing potential future quality standards and extending the shelf-life of cooked ham.

6 REFERENCES

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